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ON THE MODE OF ACTION OF THE SULFONAMIDES

III. Purines, Amino Acids, Peptones and Pancreas as Antagonists and Potentiators of Sulfonamide in E. coli

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Using strains of *Escherichia coli* which grow in simple synthetic media, Bliss and Long (1) and we (2, 3) independently showed that methionine has appreciable antisulfonamide activity. Bliss and Long also reported that arginine and lysine irregularly showed slight antisulfonamide action. In the case of our strain, no other amino acid *per se* is antagonistic, though some including ethionine are inhibitory. However, if methionine is already present in the medium, then glycine, serine, and allothreonine become antagonistic (4). It was also reported briefly (5) that certain purines can potentiate or antagonize the sulfonamides.

Although the individual actions of the foregoing agents are not great compared to that of *p*-aminobenzoic acid (6), their effects are additive and thus may reach significant levels. The question, therefore, arises whether they can account for the antisulfonamide activity of peptones (7, 8) and other biological materials (8, 9). If they can not, either alone or with *p*-aminobenzoic acid, then other inhibitory agents yet to be discovered must reside in these preparations.

The general plan of this paper is as follows. After dealing with the methods employed, we will describe quantitatively the actions of the purines, then of the amino acids. A comparison will then be made of purine-amino acid mixtures and peptone or tissue extracts. It should be emphasized that the methods employed rule out "apparent" antagonism due simply to an acceleration of growth in the control.

We shall be quite specific in our use of the terms *antagonist*, *potentiator*, and *synergist*. By *antagonist* we mean an agent whose presence increases the concentration of sulfonamide required to produce a stated inhibition (usually 50%) in the *rate* of growth. On the other hand, both *potentiators* and *synergists* decrease the required concentration of sulfonamide. When present alone (in the absence of sulfonamide), however, the potentiator is without effect, whereas the synergist by itself inhibits the rate of growth.

In addition to the agents mentioned, two others have been reported as affecting the inhibition or other coli strains by the sulfonamides. High concentrations (1.3%) of urea are reported (10) to potentiate sulfadiazine, whereas glutamic acid at 10^{-3} and 10^{-2}M antagonizes sulfanilamide (11). Unfortunately, the magnitude of these effects can not be stated quantitatively, since but a single

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concentration of sulfonamide was employed; the data indicate the glutamic acid effect to be rather small.

METHODS. Since the methods employed were previously described in detail (12), only a brief description will be given here.

Organism: Escherichia coli, no. 6522, American Type Culture Collection.

Medium SG: In one liter of distilled water were dissolved 2 grams each of glucose, KH_2PO_4 and $(NH_4)_2HPO_4$, 4 grams of NaCl, and 0.2 grams of $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.2 with NaOH and the solution was boiled and filtered if necessary. It was placed in pyrex tubes and autoclaved for 15 minutes at 20 pounds. Sulfonamides were added to the sterilized medium.

Inoculation and growth. About 150 organisms from an actively growing culture were inoculated into 7 to 8 cc. of broth, which was incubated at 37°. The time was determined for the population to reach a density of 132 million per cc., estimated by the Evelyn photoelectric colorimeter. In the uninhibited culture, growth was exponential for practically the entire period of about 22 divisions. From these data the velocity constant of multiplication K was calculated.

Assessment of inhibition. Although the multiplication rate was practically constant in the controls, it might change considerably in the inhibited cultures during the course of the 22 divisions. For practical reasons we determined the mean rate of growth of the inhibited culture for this period, from which the mean velocity constant of multiplication Km could be calculated. Usually it is more convenient to express the rate of multiplication as a percentage of the control rate. Since K and Km are inversely proportional to the time spent in growth,

percentage of control rate = control time for 22 divisions/experimental time for 22 divisions × 100

By control, in this paper, we mean a culture identical in every respect with the experimental except that it contains no sulfonamide.

Measure of antagonism and potentiation. A more or less linear relationship obtains when the growth rate is plotted against the logarithm of the sulfonamide concentration. Previous (3) and present work have shown that potentiators and antagonists, including p-aminobenzoic acid, do not change the nature of this relationship, though they do shift the curve back and forth along the abscissa (as in figure 2). Peptone is the one notable exception, but even it does not affect the shape of the curve until an inhibition of more than about 60% is achieved.

We shall therefore measure potentiation and antagonism in terms of the shift of such curves along the abscissa. This is most conveniently done by determining the concentration of sulfonamide necessary to inhibit the growth rate by 50% in the presence and absence of the substance in question. The *fold* increase or decrease in sulfonamide concentration will be taken as the measure of the antagonism or potentiation. In dealing with peptone, the change in shape of the curve will also be taken into consideration. All concentrations given are *molar*. The equivalent of 0.001 *M* in mgm. per 100 cc. for the sulfonamides used is: sulfanilamide 17.2, sulfapyridine 24.9, sulfathiazole 25.5, sulfadiazine 25.

Example. To measure the antagonism of methionine, serine, glycine and xanthine for sulfanilamide in medium SG, medium SG was made up containing these four substances. Four tubes of such medium were inoculated, containing 0, 10^{-4}, 3×10^{-4}, and 10^{-3}M sulfanilamide. These attained the endpoint of growth (132 million per cc.) in 16, 18.5, 21.5, and 53 hours, respectively. Hence the growth-rates of the inhibited cultures as *per cent control* were 86, 74, and 30, respectively. These data are plotted in figure 2, open circles. By interpolation it is found that 5.6×10^{-4}M sulfanilamide inhibited by 50%. In medium SG alone, by the same method of analysis (not graphed), it was found simultaneously that 6×10^{-5}M sulfanilamide inhibited by 50%. In this experiment, therefore, the fold antagonism was $(5.6 \times 10^{-4})/(6 \times 10^{-5})$ which equals 9.3.

PURINES. In the presence of methionine, cozymase of 30% purity showed a slight antagonism, which was enhanced by hydrolysis. It was then found that neither nicotinic acid nor its amide was active, but that various purines could serve as antagonists or potentiators, depending upon the experimental conditions. The active purines are discussed below; the inactive purines and related compounds tested for antagonism at 10^{-3} and 10^{-4}M in medium SG containing 10^{-4} *dl*-methionine are as follows:

Purines. Uric acid, theophylline, theobromine, caffeine, muscle adenylic acid.

Pyrimidines. Alloxan, alloxantin, barbituric acid, phenobarbital, cocarboxylase, cytosine, isocytosine, uracil, 6-methyl-uracil, 5-aminouracil, uramil, dialuric acid, thymine, and the following substituted pyrimidines: *2*-amino, *4*-amino, *6*-oxy, *2*-*4*-diamino-*6*-oxy, and *2*-oxy-*4*-methyl-*6*-amino.

Others. Hydantoin, allantoin, parabanic acid, pyrazine monocarboxylic acid, leucopterine and xanthopterine, ascorbic acid, inositol, pantothenic acid, pyridoxine, riboflavin, choline, thiamin chloride, *2*-methyl-*1*-*4*-naphthoquinone diacetate.

Since the results obtained with the purines are rather complex, those for sulfanilamide have been tabulated and analysed statistically in table 1. Data for the other sulfonamides are listed in table 2. Only four purines were found to be active, xanthine and guanine, on the one hand, whose behavior stood in contrast to that of adenine and hypoxanthine. In general, the addition of any or all of these substances to the medium did not change the rate of growth in the absence of sulfonamide. The tables show the following:

1. In the *absence* of methionine, all four purines are potentiators of sulfonamide action, adenine and hypoxanthine the most so.

2. In the *presence* of methionine, xanthine and guanine antagonize, whereas adenine and hypoxanthine potentiate the sulfanilamide inhibition. Thus xanthine increases by 2.1 fold the concentration of sulfanilamide required to inhibit 50%, and adenine drops the concentration to 0.33 of the original.

3. The action on the heterocyclic derivatives of sulfanilamide is about the same as on sulfanilamide itself.

None of these effects can be obtained if 1% of proteose-peptone (Difco) is added to the medium. Evidently, peptone already contains these purines in optimal amount, or contains substances which nullify their actions.

It is of some interest that these effects are quite specific. Further methylation or oxidation of the purine, as in caffeine and uric acid, abolishes all activity, nor have the corresponding pyrimidines available to us been active. The fact that xanthine and guanine contrast in their behavior with adenine and hypoxanthine shows that the antisulfonamide action is associated with the substitution at the *2*-position, whereas potentiation is determined at the *6*-position. This suggests that *2*-sulfanilamido-*6*-hydroxy-purine might be a potent chemotherapeutic agent.

Numerous experiments were performed to determine what concentration of purine is necessary to produce the antagonism or potentiation just described.

To summarize briefly, the active range for all four of the purines was found to lie between 10^{-6} and 10^{-4}M. The optimal activity probably is nearer to 10^{-5} than 10^{-4}M. The presence of purine at these concentrations did not change

TABLE 1

Sulfanilamide concentration required to inhibit growth rate 50%

The basal medium of inorganic salts and glucose was inoculated with about 20 organisms per cc; The controls required about 19 hours to complete 22 divisions and reach the end-point of 132 millions per cc.; the addition of methionine reduced this to about 17 hours.

PURINE 10^{-4}M	*dl*-METHIONINE 10^{-4}M	NUMBER OF EXPERIMENTS	MEAN LOG MOLAR SA	STANDARD ERROR	MEAN MOLAR SA*	FOLD-CHANGE†
	–	12	$\bar{5}.997$	0.035	9.9×10^{-5}	
Xanthine	–	6	$\bar{5}.783$‡	0.053	6.0×10^{-5}	0.60
Guanine	–	4	$\bar{5}.825$‡	0.075	6.8×10^{-5}	0.68
Adenine	–	3	$\bar{5}.397$§	0.032	2.5×10^{-5}	0.25
Hypoxanthine	–	4	$\bar{5}.500$§	0.047	3.2×10^{-5}	0.32
	+	11	$\bar{4}.636$	0.023	4.3×10^{-4}	–
Xanthine	+	6	$\bar{4}.966$§	0.029	9.3×10^{-4}	2.10
Guanine	+	5	$\bar{4}.912$§	0.019	8.2×10^{-4}	1.90
Adenine	+	4	$\bar{4}.155$§	0.026	1.4×10^{-4}	0.33
Hypoxanthine	+	4	$\bar{4}.130$§	0.046	1.4×10^{-4}	0.33

* The antilog of mean log molar SA.

† A *fold*-change of more than 1 indicates antagonism, of less than 1 potentiation.

‡ The difference between this concentration and that required in the absence of purine is significant by the *t*-test, *i.e.*, the probability lies between 0.05 and 0.01.

§ The difference is highly significant, *i.e.*, the probability is less than 0.01.

TABLE 2

Change in sulfonamide concentration required to inhibit growth rate 50%

Conditions similar to those in table 1. The bracketted figures after the means indicate the number of tests.

PURINE 10^{-4}M	*dl*-METHIONINE 10^{-4} OR 10^{-3}M	FOLD CHANGE		
		Sulfapyridine	Sulfathiazole	Sulfadiazine
Xanthine	–		0.51 (1)	
Guanine	–			
Adenine	–		0.21 (1)	
Hypoxanthine	–			0.77 (1)
Xanthine	+	1.80 (3)	1.70 (4)	1.80 (2)
Guanine	+	1.70 (2)	1.90 (2)	1.60 (1)
Adenine	+	0.31 (1)	0.32 (1)	0.18 (1)
Hypoxanthine	+	0.36 (1)	0.11 (1)	0.62 (1)

the fact previously established for methionine (3), that its maximal activity is shown in the range 10^{-6} to 10^{-4}M.

It should also be pointed out that the action of the purines becomes evident

only after a certain number of divisions has occurred. A typical example for adenine is shown graphically in figure 1, where the number of divisions is plotted against the time in minutes. The data were obtained by the *Ksd* technic as described previously (12). In this particular experiment, inhibition due to sulfanilamide did not appear until after the occurrence of 3.5 divisions. The decrease in growth-rate due to the adenine occurred 1 division later, and then very suddenly.

Xanthine in the presence of methionine acts as a sulfonamide antagonist, this effect first appearing after about 7 or 8 divisions. In the absence of methionine, xanthine is a feeble potentiator of the sulfonamides. Again this effect is noted only after the occurence of some 7–10 divisions.

The effect of *p*-aminobenzoic acid upon these relationships is to reduce the "effective" concentration of sulfonamide. For example, to demonstrate the

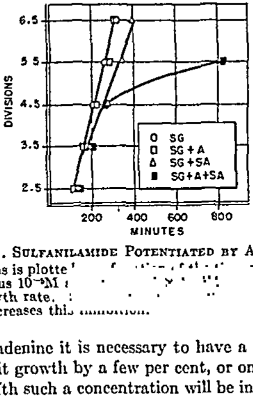

FIG. 1. SULFANILAMIDE POTENTIATED BY ADENINE

The number of divisions is plotte [illegible] medium SG, plus or minus 10^{-4}M [illegible] does not change the growth rate. [illegible] sions; adenine greatly increases this [illegible]

potentiating effect of adenine it is necessary to have a sulfonamide concentration sufficient to inhibit growth by a few per cent, or one which just falls short of doing so; and one-fifth such a concentration will be insufficient. If *p*-aminobenzoic acid is added to a just adequate concentration of sulfonamide, the adenine effect will be lost until the sulfonamide concentration is again raised so that it is inhibitory, or is on the verge of being so. The same is true for the antagonistic actions of xanthine and guanine. It is to be emphasized that apparently the purines do not affect the competition between *p*-aminobenzoic acid and the sulfonamides.

We interpret this to mean that the purines are secondary antagonists and potentiators; *i.e.*, they affect reactions which normally are not limiting factors in growth However, in the presence of sulfonamide these reactions do become limiting, and hence their increase or decrease can lead to antagonism or potentiation as described above. The general nature of such relationships was discussed in detail previously (3, 12).

It is of interest to recall that the purines are more or less essential for the growth of *Clostridium acidi-urici* and *cylindrosporum* (13), *Staphylococcus aureus* (14), *Phycomyces* (15), strain C203S, a group *A* hemolytic streptococcus (16), and *Lactobacillus plantarum* (17). The literature shows that xanthine, hypoxanthine, guanine, and adenine are the four important purines, but the active individuals of this group vary from species to species. This suggests that the purine-sulfonamide relationship will vary likewise, which we have actually found to be the case. Using strains which grow well in medium SG, we have found that *Pseudomonas fluorescens* and *Mycobacterium sp*. are sensitive to both sulfanilamide and sulfathiazole, and that *p*-aminobenzoic acid antagonizes in each case. Methionine by itself, however, has no effect. In *Pseudomonas*, adenine and xanthine definitely antagonize sulfanilamide; in *Mycobacterium*, when methionine is present, adenine and xanthine potentiate sulfanilamide.

The finding that purines affect the action of the sulfonamides *in vitro* has been confirmed *in vivo* by Martin and Fisher (18). They found adenine to antagonize the common sulfonamides when used to treat a hemolytic streptococcus infection in the mouse. Curiously, they found guanine to be without effect, although Pappenheimer and Hottle (16) stated that either guanine or adenine could serve as a growth factor.

Amino acids. It was shown previously that no individual amino acid added to medium SG could specifically affect the sufonamide inhibition, except methionine (especially the *l*-isomer). However, with methionine already added to medium SG, three showed some antagonism, glycine, serine, and allothreonine (but not threonine). Structurally these three are somewhat related, for serine is hydroxy-methyl-glycine and allothreonine is α-hydroxyl-ethyl-glycine. Serine is inhibitory *per se* at 10^{-3}M, reducing the growth rate to about one-third of the control, but it has no such effect at 10^{-4}M which is adequate to demonstrate its antisulfonamide action.

All of these actions add to those of *p*-aminobenzoic acid and the purines, and even to some extent among themselves. This is illustrated in table 3 where the fold increase (*i.e.*, antagonism) is given for glycine, serine and allotheonine alone and in various combinations. Table 4 (lines 4 and 5) shows their action with *p*-aminobenzoic acid. Looking over all of our experiments we find that the addition to medium SG of a total of 10^{-4} mols of amino acids and purine (*l*-methionine and xanthine at 10^{-5}M plus *dl*-serine and glycine at 4×10^{-5}M) will antagonize a 10–25 fold increase in sulfanilamide (about 10^{-3} mols) or in sulfathiazole (about 7×10^{-6} mols).

As a check on the conclusion that only four amino acids are active antagonists in our strain of *coli*, a mixture of all known, available, naturally occurring amino acids was prepared and tested. Two groups of amino acids made up the mixture. *Group A: dl*-alanine, *l*-arginine, *dl*-aspartic, *dl*-glutamic, glycine, *dl*-histidine, *dl*-isoleucine, *dl*-leucine, *dl*-lysine, *dl*-methionine, *dl*-norleucine, *l*-oxyproline, *dl*-phenylalanine, *dl*-proline, *dl*-serine, *dl*-threonine, *l*-tryptophane, *dl*-valine. *Group B: dl*-cystine, *dl*-tyrosine. Members of group B were present at one-half the concentration of those in group A. The tryptophane was added

after the medium had been autoclaved. Allothreonine was omitted because its natural occurrence has not been established. The data in table 4 show the following:

1. The addition of amino acids speeds up the growth rate in the controls. In medium SG, about 18 hours are required for 22 divisions. The addition of

TABLE 3

Antagonism by glycine, serine, and allothreonine

The compound to be tested was added to medium SG plus 10^{-5}M *l*-methionine Its concentration was 4×10^{-4}M. Antagonism is measured by the fold *increase* in sulfonamide necessary to inhibit the growth rate 50 per cent. The bracketted numbers indicate the number of tests averaged; when marked by an asterisk a test at 10^{-4}M is included.

GLYCINE	*dl* SERINE	*dl*-ALLOTHREONINE	XANTHINE	ANTAGONISM	
				SA	*ST*
+	0	0	0	1.7 (2)*	1.6 (2)*
0	+	0	0	1.7 (3)	1.6 (3)
0	0	+	0	1.5 (1)*	1.4 (1)*
+	+	0	0	1.9 (2)	2 0 (1)
+	+	0	+	3.8 (3)	2.8 (2)

TABLE 4

Methionine, serine, and glycine compared to all the naturally occurring amino acids

Medium SG (salt + glucose) was made up according to the following schedule The control growth rate is given as the time for 22 divisions at 37°.

	ADDED TO BASAL MEDIUM					CONTROL	TO INHIBIT 50%	
	Methionine, serine, glycine, 4×10^{-5}M each	All natural amino acids		Xanthine, 10^{-4} M	*p*-Amino benzoic acid, 10^{-4} M		*SA*	*ST*
		4×10^{-5}M each	5×10^{-4} M each					
						hours	*molar*	*molar*
1	+	0	0	0	0	16.0	7.4×10^{-4}	$5\ 6 \times 10^{-6}$
2	0	+	0	0	0	12.0	5.0×10^{-4}	4.3×10^{-6}
3	0	+	0	+	0	12 7		5.0×10^{-6}
4	0	0	0	0	+	18.1		$8\ 0 \times 10^{-6}$
5	+	0	0	0	+	15.9		4.5×10^{-4}
6	0	+	0	0	+	12.1		$3\ 7 \times 10^{-4}$
7	0	0	+	0	+	13.7		4.0×10^{-4}
8	0	0	0	+	+	18.2		7.0×10^{-5}
9	0	+	0	+	+	12.2		5.9×10^{-4}

p-aminobenzoic acid does not change this (line 4). The addition of methionine, serine and glycine reduces this to about 16 hours (line 1), and of all the amino acids to as low as 12 (lines 2 and 6).

2. Nevertheless, the antagonistic action of all the amino acids is quantitatively equal to or somewhat less than that of only methionine,[2] serine, and glycine,

[2] It should be recalled that the method of calculating the inhibitions allows for differences in the control growth-rates.

It is of interest to recall that the purines are more or less essential for the growth of *Clostridium acidi-urici* and *cylindrosporum* (13), *Staphylococcus aureus* (14), *Phycomyces* (15), strain C203S, a group *A* hemolytic streptococcus (16), and *Lactobacillus plantarum* (17). The literature shows that xanthine, hypoxanthine, guanine, and adenine are the four important purines, but the active individuals of this group vary from species to species. This suggests that the purine-sulfonamide relationship will vary likewise, which we have actually found to be the case. Using strains which grow well in medium SG, we have found that *Pseudomonas fluorescens* and *Mycobacterium sp.* are sensitive to both sulfanilamide and sulfathiazole, and that *p*-aminobenzoic acid antagonizes in each case. Methionine by itself, however, has no effect. In *Pseudomonas*, adenine and xanthine definitely antagonize sulfanilamide; in *Mycobacterium*, when methionine is present, adenine and xanthine potentiate sulfanilamide.

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The curves with *sulfanilamide* are almost but not quite parallel. Peptone is slightly better than the synthetic medium. *Conclusion:* Since methionine, serine, and glycine are in peptone, and most probably xanthine in the traces required, they account for the chief part of peptone's antisulfanilamide activity. Nevertheless, there is an unexplained residuum, which cannot be a known naturally occurring amino acid (since all have been tested) or *p*-aminobenzoic acid (its curve would be parallel).

The curves with sulfathiazole are parallel at first but markedly deviate at 25% due to the insertion of a plateau in the peptone curve. For inhibitions of less than about 65%, peptone is only slightly better than the synthetic medium, but at 75% the plateau introduces a difference of 50 fold. *Conclusion:* For inhibitions of roughly less than 65%, the antagonistic action of peptone can be attributed to its content of methionine, serine, glycine, and xanthine. At greater inhibitions, however, another factor (or group of factors) make a very great contribution; it is not a known naturally occurring amino acid or *p*-aminobenzoic acid.

The antagonist discussed above corresponds to *P-2*, defined previously (3). From a study of the *growth-rate vs drug-concentration* cruves for SG plus methionine and SG plus peptone, we argued that peptone contained two antagonists in addition to methionine, *P-1* responsible for the parallel shift, and *P-2* for the plateau. The present work indicates that *P-1* is largely, if not entirely, due to serine, glycine, and xanthine. The nature of *P-2*, however, is unknown.

p-Aminobenzoic acid content of peptone. If peptone contained *p*-aminobenzoic acid, it would be a member of the first group of antagonists (*vide supra*), responsible for the parallel shift. Analysis detailed below, however, shows peptone to contain little *p*-aminobenzoic acid, whereas we may be reasonably certain that methionine, glycine, serine, and probably xanthine are present.

Blanchard (19) has described in detail the isolation of *p*-aminobenzoic acid from yeast, which may contain as much as 0.5 mgm. %. The compound exists both in the free and in the combined states. Hill and Mann (11), without giving any details, report the common peptones to contain no *p*-aminobenzoic acid. We find that direct analysis of peptone solutions can not be performed because of the presence of interfering substances. We have, therefore, followed Blanchard's technic, extracting with ether at pH 3.8, evaporating to dryness, dissolving the material in water, and analysing by the method of Bratton and Marshall (20). Though the color thus developed appears to be of the correct tint, judged by the eye, its intensity, unlike that of *p*-aminobenzoic acid, continues to increase for 20–120 minutes. If *p*-aminobenzoic acid or sulfanilamide is added to peptone, or its ether extract, the color developed immediately corresponds to the added arylamine, and this is followed by a period of increasing intensity due to the extract *per se*.

The interpretation of such data is not possible in precise terms. For purposes of orientation we may suppose that the color developed during the first few minutes represents the maximum amount of free, unsubstituted *p*-aminobenzoic acid present. The final intensity of the color, measured in terms of the extinc-

tion coefficient of *p*-aminobenzoic acid, provides a rough measure of the total, ether-soluble, arylamine.

For our present purposes, the following two analyses are of interest. Two 10% solutions of preteose-peptone, one in water, the other in 1N NaOH, were autoclaved at 120° for twenty minutes. It was found that: autoclaved proteose-peptone contains, of *free PAB* not more than 2×10^{-9} mols per gram; of *total arylamine* not more than 2×10^{-8} mols per gram. *Hydrolyzed* peptone contains, of *free PAB* not more than 5×10^{-8} mols per gram; of *total arylamine* not more than 1.5×10^{-7} mols per gram.

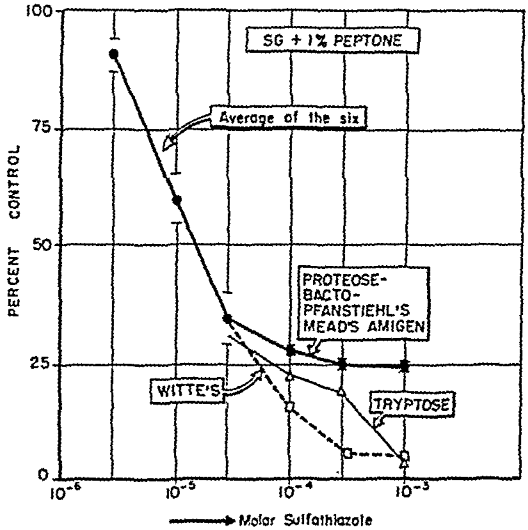

FIG. 3. ANTAGONISM BY COMMON PEPTONES

Per cent control rate of growth plotted as a function of sulfathiazole concentration for organisms grown in medium SG containing 1% of various peptones (final pH 7.2). When the plotted symbol represents the average of a number of points, the spread is indicated by the break in the ruling of the ordinate. The mean sulfathiazole concentration for 50% is 1.4×10^{-5}M.

For purposes of comparison, we have added *p*-aminobenzoic acid to medium SG alone, or containing *l*-methionine (10^{-5}M), xanthine (10^{-5}M), *dl*-serine (4×10^{-5}M) and glycine (4×10^{-5}M) together, in order to determine how much of the synthetic acid is required to raise the antisulfonamide titer of the medium to that of proteose-peptone. To avoid the plateau, the comparisons were made at 50% inhibition of the growth-rate. It was found that medium SG required the addition of 10^{-6}M *p*-aminobenzoic acid, which is far more than can be found in proteose-peptone by chemical analysis. The aminoacid-purine medium required an amount (1.5×10^{-7}M) that could be present in proteose-peptone if the total arylamine measured represents bound *p*-aminobenzoic acid which the bacteria can release, or can use as such.

It is therefore suggested that methionine, serine, glycine and purine are active in proteose-peptone, and that only a small quantity of p-aminobenzoic acid somewhat increases the total antisulfonamide action.

The behavior of different peptones. In figure 3 there are presented the curves for a variety of peptones, each tested at 1% in medium SG (pH 7.2). At such a concentration, the linear part of the curve is practically the same in all cases, though the strength of P-2 as shown by the plateau does vary. Witte's peptone has the least and is followed by bacto-tryptose (Difco). The other four tested showed no difference here. When tested at various dilutions it was found that none showed a plateau at a concentration of 0.01%. At 0.1%, Pfanstiehl's and proteose-peptone showed plateaus, whereas Witte's, Bactopeptone and Mead's Amigen solution showed none. It will be recalled that MacLeod (9) noted Witte's peptone to be a poor antagonist.

TABLE 5

Antagonism of protein digests

0.75 gram of protein (dry) plus 30 mgm. of pancreatin (Armour), plus several drops of toluene were incubated at 37° for 72 hours at pH 7.8 in phosphate buffer. The solutions were then acidified, boiled, filtered, and added to medium SG plus 10^{-5}M l-methionine and xanthine, and 4×10^{-4} M glycine and dl-serine. The concentrations specified are in terms of the original material.

CONCENTRATION		PER CENT CONTROL		
Protein	Pancreatin, U S P.	3×10^{-5} *M* ST	10^{-4} *M* ST	3×10^{-4} *M* ST
mg./cc.	*mg./cc.*			
0	0.68	34	34	31
0	2.7	24	16 (?)	25
0	13.6	32	16−	16−
Gelatine, 13.6	0.54	53	33	30
Casein, 13.6	0 54	53	39	31
Fibrin, 13.6	0.54	40	31	27

Source of P-2. If P-2 is polypeptide its production might be possible from purified protein by tryptic or peptic digestion. In carrying out such experiments, both with proteins and tissue extracts, we noted that our controls (pancreatin alone) were actively antagonistic. Quantitative comparisons revealed that the activity of our digests was due largely to the pancreatin used in their preparation. This is illustrated in table 5. The table also shows that an inhibitory factor masks the antagonist when high concentrations of pancreatin are used. We shall call this inhibitor I-1.

Factor *P-2* was prepared from fresh pig pancreas as follows. 100 grams of fresh pancreas, freed of adherent fat by dissection, was ground with 75 cc. of alcohol in a Waring Blendor, then poured into 300 cc. of boiling water plus 1 cc. of concentrated HCl. When the temperature reached 95°C., the suspension was cooled, the pH was brought to 4.5, and the liquid was filtered off through a Chardin paper. The pH was then brought to 7.8, the solution boiled, and re-

filtered. The dry weight was determined. The extract was assayed at various concentrations for antagonistic action. As shown in figure 4, in which the experimental details are given, such a pancreas extract and proteose-peptone have about the same antagonistic action against sulfathiazole. We have examined other tissues in the rabbit for activity. Spleen seems to be a poor second, followed by liver, kidney, brain and muscle, which showed little activity.

These results suggest that commercial peptones may owe at least part of their activity to their content of pancreas and spleen. Mr. William K. Fox of the Pfanstiehl Co. has informed us, however, that neither pancreas nor spleen is involved in the preparation of Pfanstiehl's peptone, which is the most potent of those we have tested. On the other hand, Mead's Amigen solution is labelled as made from pork pancreas and casein.[3] We are now attempting the further

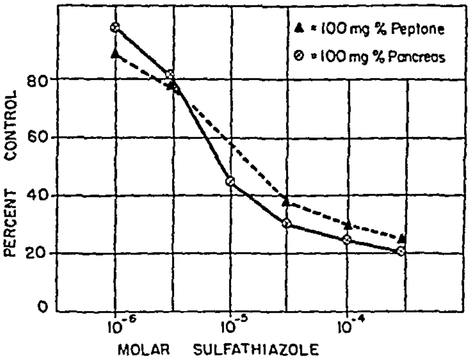

FIG. 4. ANTAGONISM OF PEPTONE AND PANCREAS COMPARED

Per cent control rate of growth plotted as a function of sulfathiazole concentration for organisms grown in medium SG containing *l*-methionine and xanthine at 10^{-5}M, and glycine and *dl*-serine at 4×10^{-5}M. To 100 cc. of this basal medium there was added 100 mgm. (dry weight) of pancreatic extract (circles), and proteose-peptone (triangles). The graph indicates that the two preparations are about equi-potent. Further dilution of either peptone or extract will cause the loss of the plateau.

purification of P-2. The facts presented thus far indicate that it is not a known, naturally occurring amino acid, a common purine, or *p*-aminobenzoic acid.

P-2 and the theory of sulfonamide action. Woods (6) postulated that *p*-aminobenzoic acid and the sulfonamides compete for an enzyme within the bacterial cell. In support of this, he pointed out that sulfapyridine, which is five times as potent as sulfanilamide, requires five times as much *p*-aminobenzoic to antagonize it. This implies that differences in potency among the sulfonamides are to be explained in terms of their different affinity constants for the enzyme in question.

During the past year a number of papers have reported detailed quantitative investigations upon the various implications of the Woods theory. Wyss (21) showed that sulfanilamide and *p*-aminobenzoic acid behave as the kinetic analy-

[3] Thus far (1 month) the Difco Laboratories have not acknowledged our inquiry.

sis predicts. Wyss, Grubaugh, and Schmelkes (22) showed that the difference in effectiveness between sulfanilamide and sulfathiazole, in their relation to *p*-aminobenzoic acid, is independent of the species of bacterium (seven species were tested) used for the experiment. Hence, they conclude the mode of action to be non-specific.

Simultaneously, papers appeared by Rose and Fox (23) and by Wood (24) *showing for different strains of coli that the same concentration of p-aminobenzoic acid* is required to antagonize the minimal effective concentration of any sulfonamide. Rose and Fox reported that although the molar drug concentrations varied from 2.5×10^{-3} for sulfanilamide to 4×10^{-6} for sulfathiazole (600-fold), the required minimal concentration of *p*-aminobenzoic acid remained constant at 5×10^{-7}. Wood expressed his data rather differently, but upon our recalculation (multiplying columns 1 and 2 of his table II) they reveal the same *fact: although the molar concentrations varied from 4×10^{-4} for sulfanila*mide to 8×10^{-6} for sulfathiazole (50-fold), the required minimal amount of *p*-aminobenzoic acid varied only from 2.5 to 2×10^{-7} (excluding diaminodiphenylsulfone).

That the sulfonamides differ greatly in their degree of ionization is an important, new consideration. Schmelkes *et al.* (25) found the potency of the drugs to increase with pH, indicating the active agent to be an anion. Fox and Rose (26) believe the differences in potency among the sulfonamides are simply a reflection of the differences in ionization. They point out that 1 molecule of *p*-aminobenzoic acid antagonizes 1.4 ions of sulfanilamide, 1.4 of sulfapyridine, 4.9 of sulfathiazole, and 6.4 of sulfadiazine. On this basis the sulfonamides are equipotent to within a factor of 5, whereas on a molecular basis it is only to within a factor of 600.

The points indicated by the foregoing may be summarized as follows. The sulfonamides inhibit only by competing with *p*-aminobenzoic acid, and do not enter directly into other reactions. Since ions are the active agents, the differences in potency among the sulfonamides are to be explained largely in terms of their different dissociation constants, and to a smaller extent in terms of their varying affinity constants for the bacterial enzyme, their ability to penetrate the bacterium, etc.

We would like to point out, however, that two pieces of evidence make it difficult to accept this attractive theory without further qualification.

(*1*) Taking the average of the constants supplied by Schmelkes *et al.* (25) and Fox and Rose (26), we have recalculated Wood's data for coli (24). It is found that 1 molecule of *p*-aminobenzoic acid antagonized 1.6 ions of sulfanilamide, 14 of sulfapyridine, 32 of sulfathiazole, and 95 of sulfadiazine. Thus on an ionic basis there is a difference in potency of 59-fold, which is practically the same as that calculated on a molecular basis (50-fold). Evidently, this strain only partially satisfies the theory outlined above. For it, on the one hand, the varying potency of the sulfonamides can not be explained merely in terms of their varying ionization. On the other hand, the constancy of the *p*-aminobenzoic acid concentration necessary to antagonize the minimal effective concen-

trations of all the sulfonamides (in peptone-free medium) suggests a single locus of action.

(*2*) The behavior of antagonist *P-2* is difficult to explain in terms of the foregoing theory. *P-2* affects the heterocyclic derivatives of sulfanilamide far more than sulfanilamide itself. Furthermore, its action becomes evident only when the growth-rate has been inhibited by about 75%. This is illustrated in figure 2, and more extensively in other data published previously (3, 12).

The use of ionic instead of molecular concentrations does not alter the situation. For example, in 2% proteose-peptone-glucose medium, pH 7.8, the ionic molar concentrations necessary to inhibit by 30% were: sulfanilamide 8×10^{-7}, sulfapyridine 4×10^{-6}, sulfathiazole 3.6×10^{-6}, and sulfadiazine 4×10^{-6} (data from (12), table 1). The agreement among these is very good, though sulfanilamide stands somewhat apart. However, to inhibit the growth-rate by 85%, the ionic molar concentrations were: sulfanilamide 8×10^{-6}, sulfapyridine 5×10^{-4}, sulfathiazole 4.3×10^{-4}, sulfadiazine 9.6×10^{-4}. Thus, judged at 30% inhibition the ionic sulfonamides were equipotent to within a factor of 5; judged at 85% inhibition, however, they were equipotent only to within a factor of 120.

Of the possible explanations, two may be mentioned First, it is possible that the heterocyclic derivatives inhibit systems in addition to that involving *p*-aminobenzoic acid; these are slightly or not at all affected by sulfanilamide. Under the usual experimental conditions, the "additional systems" become limiting factors only when the rate of growth is inhibited by more than 60–70%. Secondly, the possibility exisits that the heterocyclic derivatives react reversibly with some constituent of peptone and pancreas to form an inactive compound. The curves for growth-rate *vs.* sulfonamide-concentration would then represent the titration of the unknown agent by sulfonamide. This would be consistent with the work of Schonholzer (30) and of Davis (31) who showed that sulfonamides are bound to plasma proteins.

In conclusion it may be pointed out that the secondary antagonists fit into the theory without difficulty. Inhibition of the reaction involving *p*-aminobenzoic acid, as postulated previously, automatically will inhibit other reactions for which its products eventually serve as substrates. In the presence of inhibitory concentrations of sulfonamide, such *secondary reactions* can be reinstituted by supplying them with their missing substrates, which thus function as *secondary antagonists*. In addition, it is necessary to postulate *special reactions* which though quite independent of *p*-aminobenzoic acid systems are inhibited by at least one, but not necessarily all, of the sulfonamides. Such a system exists in *Shigella* where under rather special circumstances it is possible for sulfapyridine to compete successfully with nicotinamide (27). It is possible that *P-2* is a *special antagonist*, functioning in such a system. The same general type of theory is employed by McIlwain (28, 29) in his search for and investigation of new chemotherapeutic agents which enter into competition with essential metabolites other than *p*-aminobenzoic acid.

SUMMARY

This work, from one point of view, is an attempt to compose a synthetic medium which will duplicate the antisulfonamide activity of peptones. *E. coli* was presented with a variety of substances which probably occur in commercial peptones, and by its own response has indicated which of these can affect the sulfonamide-inhibition. The principal results are:

(1) *Naturally occurring substances may either antagonize or potentiate the* sulfonamides. It is also possible that natural synergists exist. Hitherto all discussion has centered upon the occurrence of antagonists in tissue extracts. Future work will have to decide whether the balance between antagonists and potentiators (and synergists) always favors the antagonists as, for example, now seems to be the case in necrotic processes.

(2) *The active substances in commercial peptones may now be divided into* two groups. The first group comprises four amino acids, several purines, and possibly *p*-aminobenzoic acid, and accounts for almost all of the antagonism against sulfanilamide. Against sulfathiazole (and also sulfapyridine and sulfadiazine), however, the first group accounts for almost all of the antagonism *only* when the inhibition of growth is less than about 65%. At greater inhibitions, a second antagonist *P-2*, of great power, is active.

(3) The nature of *P-2* is unknown. It is water soluble; it is not a known, naturally occurring amino acid or *p*-aminobenzoic acid. Pancreas seems to be the best source for this agent, though it is neither insulin nor a protein.

(4) The actions of *P-2* are such as to suggest that the heterocyclic sulfonamides either act at more than one locus within the bacterium, or what is perhaps less likely, form inactive complexes in the medium. The bearing of these possibilities upon current theories of sulfonamide action is discussed.

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STUDIES ON MODIFICATION OF THE MORPHINE ABSTINENCE SYNDROME BY DRUGS

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The ameliorative effect of opiates on the morphine abstinence syndrome is widely appreciated, but quantitative estimates of this phenomenon have not been used to study the relative effectiveness of opiates and other drugs. Such studies appeared to offer a possible means for investigating problems pertaining to cross-effectiveness, the influence of dose and of route of administration, the duration of action of narcotic drugs, and the therapeutic value of non-opiates. The purpose of this report is to present a method for measuring modification of the abstinence syndrome and the results of its application to a variety of drugs.

The moderately intense morphine abstinence syndrome was found to be most suitable for the purpose of such studies, since it is sufficiently uniform to permit a reasonably accurate prediction of the course it would have taken had no medication been administered. Patients (addicts) were selected for study by withholding morphine after admission until the abstinence syndrome could be demonstrated and evaluated. Suitable cases were then stabilized for at least one week on the minimal amounts of morphine required to prevent signs of abstinence. The last stabilization dose was given usually at 6:00 A.M. Observations for signs of withdrawal were made at hourly intervals from the 24th to at least the 40th hour after the last stabilization dose, and the Abstinence Syndrome Intensity (*A.S.I.*) was scored by the Point System (table 1). The 30th hour of abstinence was chosen as the time to give drugs to be tested. The preceding 6 hours, or pre-dose period (24th to 30th hour) served to furnish data from which to estimate the expected course from the 30th to 40th hour had no drug been given.

A curve of abstinence syndrome expectancy from the 24th to 40th hour was obtained from the mean data on 19 patients, 5 of whom were given no medication, 7 were given 2 cc normal saline subcutaneously, and 7 received 100 mgm. thiamine intravenously at the 30th hour (1). These patients comprise group 1 controls A three constant equation calculated by the method of least squares fitted these data reasonably well. The curve determined by this equation (Points = 20.1 + 0 762 hours − (0 0233 hours)^2) is shown in figure 1. The σ (dis.) of this expectancy curve is 0.71. The pre-dose data of the various experimental groups were plotted against the corresponding portion of this control curve If the majority of the scores fell within the σ (dis.) or could be brought within these limits by a factor of ± 2 points or less, the corrected post-dose data of the experimental groups were then plotted against the remainder of the expectancy curve. When the majority of the pre-dose data could not be brought within the σ (dis.) of the expectancy curve by the above factor, cases were added to the group until this criterion was met. A factor of ± 2 points introduces a maximum post-dose uncertainty of 8 1%

The post-dose corrected data points were connected with straight lines (figure 2) and the enclosed areas from the 31st to 33rd, 33rd to 35th, 35th to 37th, and 37th to 40th hours

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TABLE 1

Point system for measuring abstinence syndrome intensity by the day (D) or by the hour (H)

SIGNS	(D.) BY DAY		(H.) BY HOUR	
	Points	Limit	Points	Limit
Yawning	1	1	1	1
Lacrimation	1	1	1	1
Rhinorrhea	1	1	1	1
Perspiration	1	1	1	1
Mydriasis	3	3	3	3
Tremor	3	3	3	3
Gooseflesh	3	3	3	3
Anorexia (40 per cent decrease in caloric intake)	3	3		
Restlessness	5	5	5	5
Emesis (each spell)	5		5	5
Fever (for each 0.1°C. rise over mean addiction level)	1		1	10
Hyperpnoea (for each resp./min. rise over mean addiction level)	1		1	10
Rise in A.M. Systolic B.P. (for each 2 mm. Hg over mean addiction level)	1	15	1	10
Weight loss (A.M.) (for each lb. from last day of addiction)	1			

Total abstinence syndrome intensity per day or per hour is the sum of the points scored in the (D) or (H) columns respectively, with due attention to the limits.

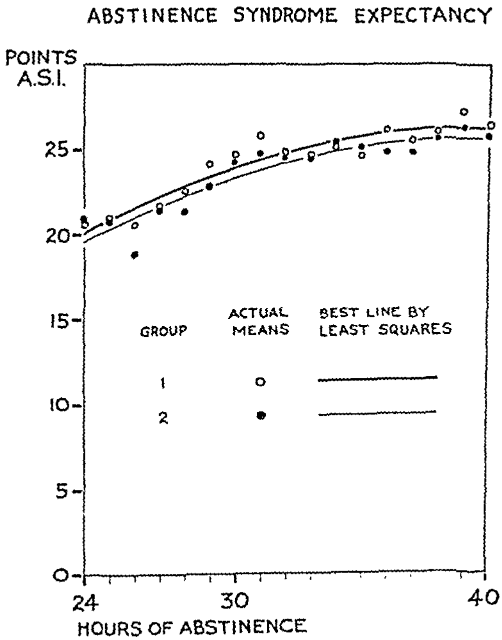

FIG. 1

were measured with a planimeter. Since any effect usually occurred rapidly, the area of the first post-dose hour (30 to 31) was not measured. Connecting these points by straight lines would not necessarily give a true picture of the change, and observations at more frequent intervals were not feasible. The drugs and doses tested and the correction factors used are shown in table 2.

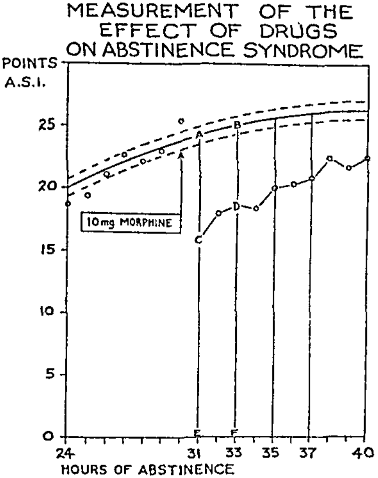

FIG. 2. Solid line is group 1 expectancy curve; dashed lines are + and − σ (dis.). Open circles are group mean data points before and after 10 mgm. morphine. 4 out of 7 pre-dose points are within σ (dis.).

Area of expectancy from 31st to 33rd hour = $AEFB$

$$\frac{CEFD \times 100}{AEFB} = \text{\% expectancy 1 to 3 hours after 10 mgm. morphine}$$

RESULTS AND DISCUSSION. The results of the effect of drugs on the expected course of the morphine abstinence syndrome are shown in figure 3 and table 2. Each graph in figure 3 depicts the post-dose effect of a drug as its percentage relationship to expectancy for the 31st to 33rd, 33rd to 35th, 35th to 37th, and 37th to 40th hours, assuming expectancy to be 100% in each period. As a result of this assumption the expectancy curve has been converted to a horizontal straight line to facilitate interpretation of the results. The results considered from the standpoint of maximum and total (31st to 40th hours) deviations from expectancy, tend to group themselves as follows:

		DEVIATIONS FROM EXPECTANCY		
Maximum, %:	+6 to −15,	−22 to −37,	−46 to −49,	>−60%
Total, %:	+5 to −11,	−16 to −23,	−34 to −41,	>−50%
	Thiamine	52 mgm. codeine	20 mgm. morphine	20 mgm. morphine i.v.
	Prostigmin	5 mgm. morphine	80 mgm. morphine-sulf ether	
	0.85% NaCl	10 mgm. morphine with or without prostigmin		
	Atropine		200 mgm. Demerol	
	Pentobarbital	104 mgm. codeine		
	No drug	100 mgm. Demerol		
	25 mgm. codeine			
	Pyridoxine			

The courses of the abstinence syndromes of patients receiving prostigmin, pentobarbital, and atropine were of the same order as those of the patients who were given normal saline, thiamine, or no drug. Hence, it would seem that prostigmin, pentobarbital, and atropine in the doses given might well be classed as placebos in so far as amelioration of the morphine abstinence syndrome is concerned.

Spies, Bean, and Ashe (2) reported that the nervousness, insomnia, irritability, abdominal pain, and weakness associated with deficiency of vitamin B_6 disappeared within four hours after the intravenous administration of 50 mgm. of pyridoxine. These symptoms also occur in the morphine abstinence syndrome. A slight but persistent reduction in abstinence syndrome intensity occurred 3 hours after administration of pyridoxine. Since no B_6 deficiency was established, the significance of this slight effect is obscure.

No. 150 (diethyl-aminomethyl-3-phenanthryl carbinol HCl) has been studied only by this method since its toxicity precluded study by the substitution technique. Mosettig and Eddy (3) reported that this synthetic compound had analgetic and other morphine-like effects on cats. By this method 150 to 200 mgm. of *no. 150* subcutaneously had definitely ameliorative effect for the first 5 hours, but this became greater from the 5th to 10th hours after its administration. The significance of this prolonged effect is not clear, for other drugs having morphine-like action showed maximum reduction in the first few hours.

All doses of morphine studied were effective and the effect increased somewhat with the dose. The dosage range was not sufficient to justify quantitative statements about this relationship. Prostigmin did not potentiate this action of morphine. The route of administration appears to be of considerable significance, for 20 mgm. of morphine intravenously produced much greater and more *sustained* effect than an equal amount given subcutaneously. While the reason for this is obscure, the result bears out the addict's preference for the intravenous route.

Morphine sulfuric ether, a possible form of conjugated morphine, and a drug which has been found to satisfy pre-formed physical dependence (4), had a striking and sustained effect. Demerol, a drug which will satisfy and produce physical dependence (5), had a marked effect.

Codeine was found to be a comparatively ineffective drug, since 26 mgm. had only a very slight and transient effect. While the larger doses caused somewhat greater and more prolonged amelioration, the extent was less than had been anticipated from substitution studies (6).

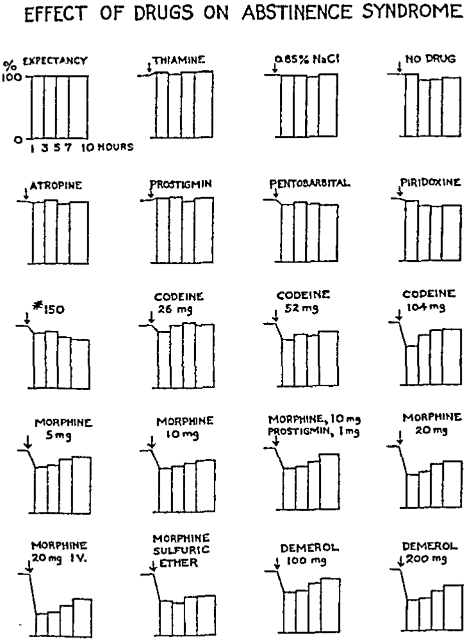

Fig. 3

These results confirm the impression that a reduction of *A.S.I.* corresponds with satisfaction of physical dependence, since those drugs which had significant effects also produce physical dependence, and since significant reductions were not obtained with drugs known to be nonaddicitve. While these results demonstrate the cross-effectiveness of addictive drugs, their relative effectiveness was usually less than that found by the substitution method. This might be expected, since cross-effectiveness is generally not complete immediately after

substitution, physical dependence usually being more adequately satisfied after a few days of substitution than promptly thereafter.

At the outset it was anticipated that this method would be useful for measuring the duration of action of addictive drugs, i.e. from the time of their administration to the time of return of *A.S.I.* to expectancy. However, it was found that

TABLE 2

DRUG	DOSE	ROUTE	NO. OF PATIENTS	CORRECTION FACTOR	DEVIATIONS FROM EXPECTANCY: Maximum	DEVIATIONS FROM EXPECTANCY: Total
	mgm.			*points*	*per cent*	*per cent*
Non-addictive:						
No drug	0	0	5	0.0	−10	−7
0.85% NaCl	2 cc.	S.C.	7	0.0	−3	−1
Thiamine	100	I.V.	7	0.0	+6	+5
Atropine	0.85–1.28	S.C.	7	+1.2	−5	−2
Prostigmin*	1	S.C.	8	+0.8	+5	+3
Pentobarbital	100	S.C.	6	−0.4	−10	−8
Pyridoxine	50	I.V.	6	−0.9	−13	−11
Unclassified:						
#150	150/200	S.C.	6	−0.4	−22	−16
Addictive:						
Codeine	26	S.C.	6	0.0	−11	−1
Codeine	52	S.C.	13	+1.4	−25	−18
Codeine	104	S.C.	6	−0.9	−37	−20
Morphine	5	S.C.	5	+1.0	−27	−18
Morphine	10	S.C.	10	+0.3	−29	−23
Morphine + Prostigmin	10 / 1	S.C.	7	0.0	−33	−23
Morphine	20	S.C.	7	+0.3	−46	−34
Morphine	20	I.V.	6	−0.5	−64	−53
Morphine sulfuric ether†	80	I.V.	6	−0.5	−47	−41
Demerol‡	100	S.C.	5	+2.0	−34	−23
Demerol	200	S.C.	6	+1.5	−49	−39

I.V. = intravenous; S.C. = subcutaneous.

* Prostigmin methyl sulfate was furnished by the Hoffman-LaRoche Company.

† Morphine sulfuric ether was prepared by Dr. L. F. Small, Head Chemist, National Institute of Health.

‡ Demerol was furnished by the Winthrop Chemical Company.

after narcotics the *A.S.I.* does not return to expectancy within 18 hours. Since this suggests a sustained therapeutic effect in addition to temporary alleviation, and since it does not appear possible to separate these effects, measurement of physical dependence action of narcotic drugs by this method did not seem justified.

In view of the ineffectiveness of prostigmin, pentobarbital, and atropine, the data on patients receiving these drugs were combined with the data on group

1 controls, and the best curve by the method of least squares was redetermined. The new curve determined by this equation (Points = 19.6 + 0.74 hours − (0.022 hours)^2) is shown in relation to the curve for group 1 controls in figure 1. The σ (dis.) for this curve is 0.83. Since the expectancy curve for the enlarged group (group 2 controls) is uniformly only 2% less than the curve for group 1 controls, and since it comprises data on a much larger group (40 patients), we propose to use it as the basis for future studies of this nature.

SUMMARY

The morphine abstinence syndrome of moderate intensity is sufficiently uniform from the 24th to the 40th hour to permit reasonably accurate prediction of its course from the 30th to 40th hour from data obtained during the preceding 6 hours. A method is described for measuring the effect of drugs administered at the 30th hour of abstinence as percentage deviations from the expected course of the abstinence syndrome. Results by this method confirm the ineffectiveness of thiamine and indicate that prostigmin, pentobarbital, and atropine in the doses given do not ameliorate abstinence syndrome intensity appreciably. Codeine in 26 mgm. dosage also was ineffective. Pyridoxine caused a slight but presumably non-significant reduction. Diethyl-aminomethyl-3-phenanthryl carbinol caused a prolonged and probably significant reduction. Significant reductions were caused by 52 and 104 mgm. codeine; 5, 10, and 20 mgm. morphine; 100 and 200 mgm. Demerol; and by 80 mgm. morphine sulfuric ether. The extent of ameliorative effect increased somewhat with dosage. Prostigmin did not potentiate this effect of morphine. The effect of 20 mgm. morphine intravenously was greater and more *sustained* than an equal amount given subcutaneously. While these results confirm the cross-effectiveness of drugs structurally or pharmacologically similar to morphine, the dose relationships were not the same as found by substitution. Although this method probably will not be useful for studying the duration of action of drugs, the technique offers a quick method for detecting probable addiction liability of drugs.

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NUTRITIONAL DEGENERATION OF THE OPTIC NERVE IN RATS: ITS RELATION TO TRYPARSAMIDE AMBLYOPIA

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The mechanism of the production of the amblyopia which may occur in humans following the use of tryparsamide has always been obscure (1). The facts that it may appear with suddenness on relatively small doses of the drug (1 or 2 grams) and that about 75% of the reactions occur within the first ten injections (2) have been unsatisfactorily explained by the concept of individual "sensitivity" (1). The exact role of syphilis, which itself may produce optic nerve atrophy, is not known. It is established, however, that tryparsamide amblyopia can occur in the absence of syphilis, as shown by Pearce in African natives infected with trypanosomiasis (3). The problem assumes increasing importance with the greater use of the drug at the present time. More neurosyphilitics are being discovered as routine serologic testing becomes more prevalent, and there is an increased demand for the drug in the treatment of trypanosomiasis. The therapeutic value of tryparsamide in the treatment of neurosyphilis is so great that were it not for ocular reactions, the drug could be used with benefit to a much greater extent than it is at present.

Although extensive studies have been made on the general toxicity of tryparsamide for animals and man, only three studies have been done on its seemingly selective effect upon the optic nerve. Young and Loevenhart (4) reported the finding of retinal hemorrhages and filling of the physiological cup in the eyes of rabbits treated with sublethal doses of tryparsamide. Lazar (5) examined the optic nerves of three rabbits which had received tryparsamide and concluded that no changes due to the drug could be demonstrated with certainty.

The production of amblyopia and optic nerve atrophy in animals, comparable to the changes which may occur in man, has been reported only recently by Longley (6) and his colleagues. These workers gave tryparsamide in ascending dosage to monkeys and were able to produce blindness in a three to four month period. However, extremely large doses of the drug (up to 0.6 gram to 0.9 gram per kgm. each week) were used to produce this effect.

Recently there has been much interest in the subject of the nutritional aspects of disease of the central nervous system. Certain observations have been made which may have a bearing on the problem of tryparsamide amblyopia. Moore (7), in South Africa, has reported the occurrence of a syndrome of marked loss of vision followed by optic atrophy among native scholars subsisting on a very inadequate diet. Excellent therapeutic results were obtained by the use of cod-liver oil and marmite. The author believes that an inadequate supply

of the vitamin B complex is the cause of the syndrome but the role of vitamin A is not completely excluded. Scott (8) described a somewhat similar syndrome in the West Indies associated with "central neuritis."

Non-syphilitic primary optic atrophy associated with pernicious anemia has been reported by Kampmeier (9), and retrobulbar neuritis in the same condition by Paufique (10), by Talbot (11), and by Hagedoorn (12). We have recently seen a patient who had had an advanced tabes dorsalis of many years standing and who developed a rapidly progressive primary optic atrophy coincidentally with the appearance of pernicious anemia and combined system disease. The relevancy of observations on pernicious anemia to the question of nutritional blindness might be questioned but the disease is, in a sense, one of abnormal nutrition.

Optic neuritis has been reported both in beri-beri and in pellagra by a number of observers. In beri-beri, like the syndrome observed by Moore, there is some question as to whether the lack of some part of the B-complex is the sole cause of the amblyopia (13). Many of these cases are probably the result of a mixed deficiency and respond completely only when vitamin A as well as B-complex are supplied. Hagedoorn (12) has reported optic neuritis which he considers to be due to abnormal nutrition, in diabetics.

Most of these clinical reports of disease of the optic nerve, associated with abnormal nutritional states, deal with supposed deficiencies of some part of the B-complex or a combined deficiency of both B-complex and vitamin A. In the experimental animal the majority of the reports deal with deficiencies of vitamin A. As far as is known to us, the production in animals of optic nerve degeneration due solely to B-complex deficiency has never been reported, although degeneration of the spinal cord and peripheral nerves on such diets has been reported by numerous observers (14, 15, 16).

On rations poor or deficient in vitamin A, blindness and/or degeneration of the optic nerve has been observed in dogs (17), in swine (18), in calves (19), in rabbits (20), and in rats (16). There are some differences of opinion as to the exact mechanism of the production of the damage. Moore (19) and his co-workers believe that, in calves, at least a part of the process is due to constriction of the optic nerve resulting from faulty bone development. However, Phillips and Bohstedt were able to produce essentially the same picture in rabbits without any accompanying stenosis of the bony foramen. In any event all are in agreement that a deficiency of vitamin A is the primary cause of the ocular disease.

The relationships of the nutritional state upon the effect of substances sometimes harmful to the optic nerve has been investigated both clinically and in the experimental animal. Carrol (21) has shown that toxic amblyopias, formerly thought to be due directly to alcohol or tobacco, can be relieved completely by the administration of large amounts of the B-complex, despite the continued use of large amounts of alcohol and tobacco. Grosz (22) and Johnson (23) have both reported similar results.

In 1939, Muncy (24) reported a series of patients with tryparsamide ambly-

opia whom he had treated with thiamine chloride and brewer's yeast tablets. He felt that the results were good and were attributable to the therapy. Exactly opposite conclusions were reached by Leinfelder and Stump (25). These workers are unable to find any demonstrable beneficial effect on the visual field changes of two patients with tryparsamide amblyopia, from the daily administration of 30 mgm. of thiamine hydrochloride.

In reviewing our own clinical material, we noted that six of the seven diabetics who received tryparsamide, developed visual reactions to the drug. This is so strikingly high an incidence of reaction, (our normal incidence is 25% of patients receiving the drug (2)) even in such a small series, that it is probably more than a coincidence. It is known that the vitamin A metabolism may be impaired in diabetes mellitus (26).

Degeneration of the spinal cord of puppies can be produced by depriving them of vitamin A, as shown by Mellanby (17). He has shown that when ergot was fed to these A-deficient animals the degenerative changes were hastened and intensified. On the other hand, if the animals were fed a diet rich in some source of vitamin A the degeneration was diminished or prevented entirely, even though similar amounts of ergot were supplied.

The only experimental work which deals directly with this problem of the effect of various poisons upon the optic nerve in animals on low vitamin intakes has been reported by Imachi and Maruo (27). In their first experiments, using CS_2, H_2S and SO_2 as poisons, they produced degneration of the optic nerve in normal rats, in rats on a poor vitamin A diet, and in animals maintained on poor intakes of vitamins B-1 and "B-2," respectively. Although some slight changes were demonstrable in the optic nerves of all four groups, the only striking degenerative changes were found in the animals maintained on the A-poor regime. The changes in the other three groups, including the normal controls, were indistinguishable from one another. In their second series of experiments they used as poisons CO, CO_2, NH_3 and emulsions of tubercle bacilli, pneumococci, staphylococci, streptococci and colon bacilli which had been killed with carbolic acid. With the exception of the ammonia, none of these poisons produced any damage in the optic nerves of the control animals. In two groups kept on A-poor and "B"-poor diets, all of these poisons produced optic nerve degeneration. The changes were much more marked in the group maintained on the vitamin A-poor regimes, however, the optic nerves of the "B"-poor animals showing either little or nor changes. In their third group of experiments they tried the effect of the overfeeding of vitamins A, "B," and C to three groups of rats which were treated with the poisons which had produced nerve degeneration even in the normal animals (CS_2, H_2S and SO_2). In the animals treated with CS_2 the optic nerve degeneration was definitely of a lighter grade in the vitamin A and "B" treated animals. No marked effects of overfeeding with vitamin C were found. The optic nerve damage produced by the H_2S and the SO_2 was not appreciably diminished by the overfeeding of either vitamins A, "B", or C.

These authors suggest that the so-called "beri-beri amblyopia" may be the

result of a poisoning while on a low vitamin A intake rather than being caused by a vitamin B deficiency. They also suggest that workers employed in the artificial silk industry be supplied with diets rich in vitamin A because of their exposure to CS_2, H_2S and SO_2.

It is hard to determine the exact dietary factor concerned in these various clinical observations, as the deficiency is almost always a multiple one and hence treated with all the known vitamins at once. The results in the experimental animals are also not free from conflict but it can be taken as established that the proper utilization of at least two dietary factors is necessary for the maintenance of the integrity of the nervous system in animals. These two essential substances are vitamin A and some fraction or fractions of the B-complex which is not thiamine, riboflavin or nicotinic acid.

Because of this clinical and experimental evidence of the importance of vitamin nutrition in disease of the optic nerve, and particularly because of the evidence of its role in the prevention of nerve damage due to poisons, it was felt that an experimental study of tryparsamide from this standpoint would be worthwhile.

PART I. VITAMIN B-COMPLEX.

Experimental materials and methods. Young growing rats of both sexes (average weight = 75 grams) were used because of the increased demand for vitamins during growth. A hybrid strain of rat bred from the Wistar Institute and Cold Spring Harbor strains was used. Animals on identical diets were caged together in groups of five and were allowed access to the diet *ad libitum*. The average daily intake of diet per rat was 10 grams. At weekly intervals the animals were weighed and examined for signs of ocular disease, or of abnormalities of gait, skin or fur, and were observed for signs of lethargy and weakness Tryparsamide in distilled water was administered through the tail veins in dosage of 0.10 grams per kgm. at weekly intervals for ten weeks This dosage was chosen on the following basis In the treatment of neurosyphilis in humans the usual single dose of tryparsamide is 3 0 grams for a 60 kgm. man or approximately 0.05 gram per kgm Because of the fact that optic nerve damage in man can occur from much smaller amounts than this it was felt to be important, in planning the experiments in rats, to use a dose which might be expected to produce damage to the optic nerve yet would not produce symptoms of general toxicity Thus 0.10 grams per kgm., though twice the per kgm dose in man, is much less than the maximum tolerated dose in rats, which is 0.6 gram per kgm. (28).

At the conclusion of the experiment the animals were killed by intraperitoneal injection of veterinary nembutal. The optic nerves from the eyeball to the chiasm were removed and fixed in 25% chloral hydrate Transverse sections of each nerve were made at a point about 0 5 cm. proximal to the eyeball and were stained both by Cajals' silver impregnation technique and Masson's trichrome stain.

A *basic synthetic diet* completely deficient in all the members of the B-complex was used for all animals. This was supplemented, in various groups, with crystalline vitamins, desiccated liver or irradiated brewer's yeast. The basic diet consisted of Casein (vitamin-free) 18%; Dextrose 68%; Salt Mixture (U.S P.) 4%; Cod liver oil 2%; Crisco 8%.

Supplements added to the basic diet. The "full crystalline" supplement consisted of the available members of the B-group of vitamins in crystalline form in the following dosage per 1,000 grams of diet: Thiamine hydrochloride 0.008 gram; Riboflavin 0 008 gram; Pyridoxine 0.008 gram; Nicotinic acid 0.080 gram; Calcium Pantothenate 0.080 gram; and Choline Chloride 1.0 gram.

The "½ crystalline" supplement consisted of ½ of the above doses of all of these vitamins.

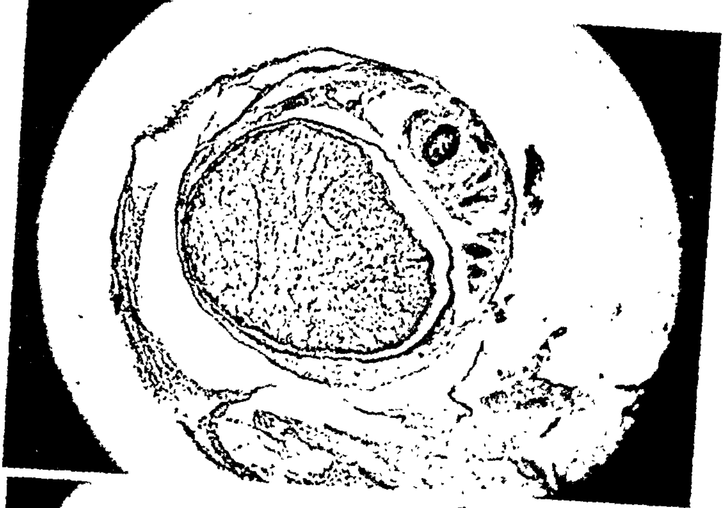
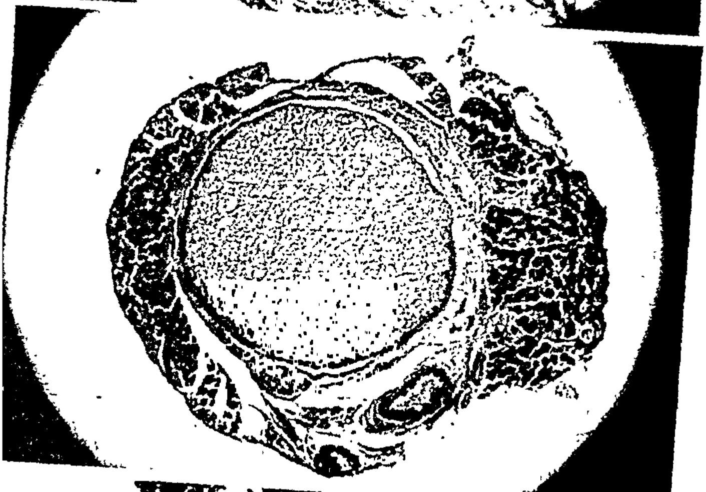


Plate 1



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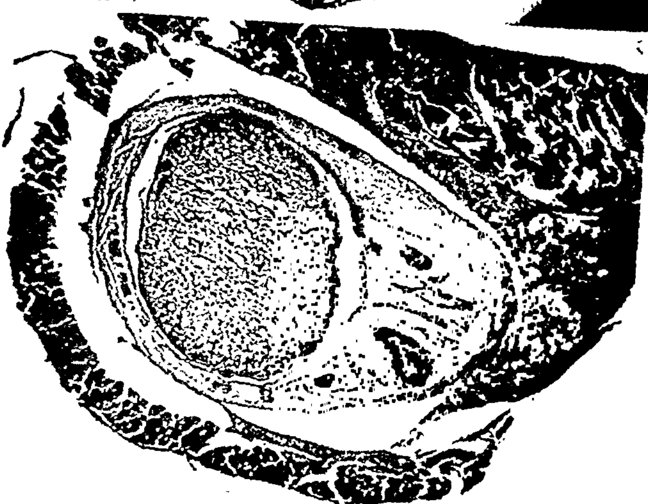

Plate 3

The *control diet* consisted of the basic B-deficient diet plus 100 grams of powdered brewer's yeast per 1,000 grams of diet. The "*one half yeast*" was exactly the same save the yeast content was only 50 grams. The *pantothenic acid deficient* diet was exactly the same as the "full crystalline" diet except for the dose of calcium pantothenate, which was 0.004 gram per 1,000 grams diet. (During the first four weeks of this experiment, two other diets were used. These consisted of the basic deficient diet plus small amounts—500 mgm. and 250 mgm.—of desiccated raw liver per 1,000 grams of diet, amounts insufficient to prevent evidence of B-complex deficiency.)

The "full crystalline" diet of course lacks some of the members of the B-complex such as inositol, biotin, folic acid and *p*-amino benzoic acid, extrinsic sources of which may be necessary to the proper nutrition of the rat. The crystalline vitamins were suspended in 50 cc. of 95% ethyl alcohol and added to the diet before mixing.

Eleven separate groups were formed. All animals, both tryparsamide treated and untreated, were placed on the respective diets for a three week period before the drug was started.

Individual experimental groups. Each group consisted of five animals.

Group 1—B-complex deficient diet.

Group 2—B-complex deficient diet plus Tryparsamide 0.1 gram/kgm. for 10 weeks.

Group 3—B-complex deficient diet—13 weeks; 0.5 gram liver per 1,000 grams diet first 4 weeks (grossly inadequate B); 100 grams yeast per 1,000 grams diet last 9 weeks (adequate).

Group 4—B-complex deficient diet—13 weeks; 0.5 gram liver per 1,000 grams diet first 4 weeks; 100.0 grams yeast per 1,000 grams diet last 9 weeks; Tryparsamide 0.10 gram/kgm. for 10 weeks.

Group 5—B-complex deficient diet;
0.25 gram liver per 1,000 grams diet first 4 weeks; 50.0 grams yeast per 1,000 grams diet last 9 weeks (½ adequate); Tryparsamide 0.1 gram per kgm. for 10 weeks.

Group 6—B-complex-deficient diet—"full crystalline" supplement (per 1,000 grams of diet): Thiamine Hydrochloride 0.008 gram; Riboflavin 0.008 gram; Nicotinic acid 0.08 gram; Calcium Pantothenate 0.08 gram; Pyridoxine 0.008 gram; Choline chloride 1.0 gram.

Group 7—B-complex deficient diet—"full crystalline" supplement—same as Group 6 plus Tryparsamide 0.10 gram per kgm. for 10 weeks.

Group 8—B-complex deficient diet—"½ crystalline supplement" (per 1000 grams of diet): Thiamine hydrochloride 0.004 gram; Riboflavin 0.004 gram; Nicotinic Acid 0.04 gram; Calcium Pantothenate 0.04 gram; Pyridoxine 0.004 gram; Choline chloride 0.5 gram; Tryparsamide 0.10 gram per kgm. for 10 weeks.

Group 9—B-complex deficient diet plus 100 grams powdered yeast per 1,000 grams diet

Group 10—B-complex deficient diet—(Pantothenic Acid poor): same as Group 7 except Calcium Pantothenate 0.004 gram per 1000 grams of diet (instead of 0.08 gram).

Group 11—B-Complex deficient diet—(Pantothenic Acid poor): same as Group 10 except no tryparsamide was given

Results (table 1). The control animals (Group 9) gained weight and were apparently completely normal all through the period of the experiment. The animals in Groups 1 and 2 (completely deficient), after an initial period of ap-

FIG. 1. VITAMIN B-COMPLEX EXPERIMENTS

Plate 1: Normal Control: Cross section of optic nerve from animal in Group 9 on completely adequate diet (Cajal silver impregnation).

Plate 2: Cross section of optic nerve from animal in Group 1 (B-complex deficient diet—

... from animal from Group 2 (B-complex deficient ... 10 weeks) showing degeneration of a more exten-

parently normal growth, developed signs of vitamin B deficiency. This was characterized by increasing lethargy, weakness, emaciation and marked involve-

TABLE 1

Rats on altered vitamin B-complex diets

GROUP	DIET	DRUG	CLINICAL EVIDENCE OF VITAMIN DEFICIENCY	OPTIC NERVE DEGENERATION (HISTOLOGIC)
1	B-complex deficient	None	Present	Advanced
2	B-complex deficient	Tryparsamide 0.1 gm. per kilo for 10 weeks	Present	Extremely advanced
3	B-complex deficient 4 weeks Adequate B-complex 9 weeks	None	Present, but disappeared after change to a diet adequate in B-complex	None
4	B-complex deficient 4 weeks B-complex adequate 9 weeks	Tryparsamide 0.1 gm. per kilo for 10 weeks	Present, but disappeared after change to diet adequate in B-complex	None
5	B-complex deficient 4 weeks B-complex ½ adequate 9 weeks	Tryparsamide 0.1 gm. per kilo for 10 weeks	Present but disappeared after change to diet adequate in B-complex	None
6	B-complex deficient Full "Crystalline Supplement"	None	None	None
7	B-complex deficient Full Crystalline Supplement	Tryparsamide 0.1 gm. per kilo for 10 weeks	None	None
8	B-complex deficient ½ Crystalline Supplement	Tryparsamide 0.1 gm. per kilo for 10 weeks	None	None
9	B-complex adequate	None	None	None
10	Pantothenic acid poor	None	Transient and minimal signs	None
11	Pantothenic acid poor	Tryparsamide 0.1 gm. per kilo for 10 weeks	Transient and minimal	None

ment of the fur, which became very scrawny and matted. No ocular signs developed, and the animals, although obviously weak, showed no abnormalities

of gait. Once the deficiency appeared, its progression was rapid. Eight of ten animals in the two groups died between 5½ to 12 weeks after starting the diet; the other two animals in Group 1 (no tryparsamide) survived to the end of the experiment but were moribund at that time.

The animals in Groups 3, 4 and 5 (almost completely B-deficient), after a two to three week period of initial growth, lost weight and developed signs of B-deficiency exactly similar to the animals in Groups 1 and 2. These animals received three weekly injections of tryparsamide after the frank signs of deficiency had appeared. Their diet was then supplemented with yeast, the signs of deficiency disappeared and they rapidly regained their lost weight and continued to grow as well as the normal controls. (Average weights at the end of the experiment for these three groups were 278, 196 and 277 grams, respectively, while Group 9 normal controls = 276 grams.)

In Groups 6, 7, 8, 10 and 11, in which the basic diet was supplemented by the crystalline members of the vitamin B group given in varying doses, the animals continued to grow as well as the normal controls throughout the entire fourteen week period. In fact the most rapid growth and greatest weight increase among the entire eleven groups was in Group 6, in which the diet, although supplemented with the crystalline vitamins, lacked the other possibly important growth factors present in yeast. Thus, with the exception of the rats which were allowed to remain on the completely B-deficient diet, all the animals in all groups showed growth rate and weight gain comparable to the normal controls.

There was no demonstrable difference in the rate and amount of weight gain between the groups which received tryparsamide and the corresponding control group. No obvious evidence of blindness was noted in any of the animals in groups 3 to 11, inclusive. Several of the rats in both groups on the completely B-deficient regime appeared to be blind shortly before death. However, these animals were so emacated and obviously weak at this stage that an estimation of the status of their vision was little more than a guess.

Histologic study of the optic nerves. Examination of sections of the optic nerves from the animals in Group 1 (completely B-deficient—no tryparsamide) showed definite evidences of extensive degeneration. Instead of the normal compact appearance, the nerve tissue appeared loose and edematous with the nerve fibers pushed apart. In cross sections sharp differentiation of the individual axone fibers could not be made out. There was a granular appearance to the tissue due to the fragmentation of the individual axones. In areas cut tangentially or longitudinally this fragmentation of the axones was striking, giving the individual fibers a "beaded" appearance. Instead of the clear area normally seen around each axone on cross section with this technique, there was great variation in the relation of the axones to each other. Some apparently had collapsed against each other, others had been pushed apart. In addition to the loose ill-defined spaces throughout the nerve there were the well-defined vacuoles which were definitely increased in size and in number. These were thought to be the oligodendria cells swollen as a result of increased ingestion of

debris, but by this technique they are not definitely demonstrable as such. The fibrous framework of the nerves was distorted and fragmented in some areas. There was increased gliosis.

Sections from the animals in Group 2 (completely B-deficient diet—tryparsamide treated) showed the same type of degeneration of the optic nerves as was seen in the untreated animals. However, the process was more extensive in these tryparsamide-treated rats. The most striking difference between the two groups, in these sections, was seen in the extent of the loose edematous appearance. This was much more marked in the sections from Group 2. In some sections the process was so extreme that the nerve tissue appeared almost completely disintegrated. All the axonal changes noted in the Group 1 sections were seen here but the amount of fragmentation and distortion was more pronounced.

No degenerative changes were demonstrable in the sections taken from the optic nerves of the animals in Groups 3 to 11, inclusive, both tryparsamide-treated and untreated.

The sections from the animals which had received the drug during a period of clinical deficiency, later cured by yeast, showed no evidence of previous damage (table 1).

Discussion. Tryparsamide in moderately large but non-toxic doses given over a 10 week period failed to produce demonstrable damage to the optic nerve in animals maintained on a synthetic diet adequate in all essentials save for the B-complex, but supplemented with 10% brewer's yeast. Animals maintained on the same purified diet, *but with no supplemental B-complex, developed degeneration of the optic nerve whether tryparsamide was administered or not. The nerve degeneration in these B-deficient animals was, however, definitely more marked in the animals which had been treated with the drug.*

Animals which had received tryparsamide during a period of frank clinical B-deficiency, but which had later been cured of the B-deficiency by supplemented yeast, showed no evidences of optic nerve degeneration seven weeks later, although the administration of the drug had been continued.

These results fit in with the current concept that some substance (or substances), present in rich sources of vitamin B such as yeast, is essential for the maintenance of the integrity of the nervous system, in this case the optic nerve. There is also some indication that when this protective substance (or substances) is lacking, a drug (tryparsamide) may intensify the damage, since controls maintained on adequate, or subadequate but not deficient diets, developed no optic nerve degeneration on similar amounts of the drug. Whether the degenerative process produced by the deficiency and aggravated by the drug can be reversed, cannot be determined from these experiments. No "sample" autopsies were done at the time of the outspoken deficiency on animals that were later treated with yeast.

From the failure to find any changes in the optic nerves of the animals in Groups 6, 7 and 8, it would seem that the substance in yeast responsible for the preservation of the nerve is one (or some) of the already isolated members of the B-complex. These animals were maintained on a diet completely lack-

ing in vitamin B but supplemented with known adequate and sub-adequate amounts of the crystalline vitamins: thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate and choline chloride. Despite the fact that their diet was deficient in the other growth substances present in yeast, these animals continued to show a normal growth rate and appearance and suffered no degeneration of the optic nerves whether or not they had received tryparsamide.

From the earlier work with relatively crude substances, it was shown that the substance present in yeast and liver, the lack of which caused nerve degeneration in chicks and pigs, was not thiamine, riboflavin or nicotinic acid but was something present in the so-called "filtrate-factor" of liver (14). When pantothenic acid was isolated and synthesized it became evident that many of the changes, other than the nervous system changes, characteristic of "filtrate-factor" deficiency, were in fact due to a lack of pantothenic acid (29). It was with the hypothesis that pantothenic acid alone might be the factor essential for the nervous system, that our experiments with Groups 10 and 11 were performed.

Because of the fact that 21 day old rats deprived completely of pantothenic acid will die in a period of 25 to 60 days, as shown by Unna and his colleagues (30), we decided to furnish minimal but grossly inadequate amounts of calcium pantothenate in an effort to permit the animals to survive for a longer period. The basic B-deficient diet was supplemented, as described above, with adequate amount of the crystalline preparations of the other vitamins. The calcium pantothenate content of this diet was 0.004 grams per 1,000 grams of diet, giving an average daily intake per rat of 40 micrograms of pantothenate. In Unna's (31) experiments this intake of calcium pantothenate, while sufficient to maintain life, was associated with the development of minimal clinical signs of deficiency in periods of four to six weeks. Because our animals were not individually caged the exact daily intake per rat is not known and exact comparisons with Unna's data cannot be made on this point. However, since all the animals had similar growth curves and since the amount of calcium pantothenate in the diet was so restricted over a thirteen week period, it seems certain that the animal's deprivation of this substance was marked. Bacterial synthesis of pantothenic acid in the intestine of the rat has been reported (32) but the amount of the products of such synthesis that has been utilized has not been sufficient to prevent pantothenic acid deficiency in animals on a limited pantothenic acid intake.

After a four week period on the diet the animals showed a slight roughening and coarsening of their fur, and appeared to be somewhat lethargic. These abnormalities had completely disappeared at the end of the sixth week, a finding similar to Unna's (31). The "bloody whiskers" described by Unna with more drastic restriction of pantothenic acid did not appear in our animals, and there were no detectable abnormalities in gait or vision.

The facts that the sections of the optic nerves showed no evidence of degeneration in either the treated or untreated group, and that sections of the spinal

cord also appeared to be entirely normal, is of interest when considered with the work of Chick (33) and her associates and the recently reported experiments of Wintrobe (34).

The former observed epileptiform seizures in rats maintained on synthetic diets completely deficient in pyridoxine. Yet histologic examination of the brain and spinal cord of three of their animals who had been on the B-6 free regime for 20–30 weeks, showed no abnormalities. Our results, when regarded with those of Chick, lend support to the recently reported conclusion of Wintrobe (34), based on his experiments with pigs, that the "antineuritic vitamin" is actually the two substances, pantothenic acid and pyridoxine. Experiments are now in progress to study the effect of this combined deficiency on the subject of tryparsamide toxicity.

PART II. VITAMIN A. The following experiments were undertaken to see if alterations of the vitamin A intake of rats had any effect on their susceptibility to tryparsamide as measured by histologic changes in the optic nerve.

Material and methods. The details of procedure in regard to rats, cages, administration and dosage of tryparsamide, were exactly similar to those described in the experiments with vitamin B. A synthetic purified diet was used which was completely lacking in vitamin A but contained all other substances known to be essential for the normal nutrition of the rat. The *basic diet* consisted of: Casein, vitamin free 18%; Cornstarch 65%; Salt Mixture (U.S.P. XI) 4%; Crisco 5%; and Dried brewer's yeast (irradiated) 8%.

This A-deficient diet was supplemented in the individual experiments with vitamin A added to the diet in cod liver-oil in three levels of dosage designated as "suboptimal," "optimal" and "excess" A. The "optimal vitamin A" diet consisted of the basic A-free diet plus 20 grams of cod liver oil (600 U per gram) per 1,000 grams of diet. The suboptimal A supplement was 0.2 grams cod liver oil per 1,000 grams of diet and the "excess A" diet consisted of 80 grams of cod liver oil per 1,000 grams of diet.

A rough approximation of the daily intake per rat of these three supplements would be: "optimal" = 120 U per 125 gram rat; "suboptimal" = 1.2 U per 125 gram rat; "excess" = 980 U per 125 gram rat. The minimal daily vitamin A requirement of the growing rat is 18 to 22 U.S.P. Units per kilo of rat (35). The 0.2 gram dose of cod liver oil was diluted with mazola before adding it to the dry mix.

The dosage of tryparsamide was identical with that used in the vitamin B experiments, i.e., 0.10 grams per kilo at weekly intervals for a total of ten injections.

All the animals were maintained on the synthetic diets for a period of four weeks before the tryparsamide was started. In 200 rats on a vitamin A-free diet from weaning, Goss and Guilbert (35) found that the average period necessary to produce depletion was 52 days. Therefore in a fourteen week experiment, with the four week period on the diet before starting the tryparsamide, there was a five or six week period of treatment with the drug after the vitamin A stores of the animals had been exhausted.

Individual experimental groups. (All groups = 5 rats each.)

Group 1—A-deficient diet + tryparsamide
Group 2—A-deficient diet
Group 3—"excess A" + tryparsamide
Group 4—"suboptimal A" + tryparsamide
Group 5—"optimal A" + tryparsamide

One week after the last injection of tryparsamide the animals were sacrificed and sections of the optic nerves were prepared, using the Cajal impregnation and the Masson trichrome techniques.

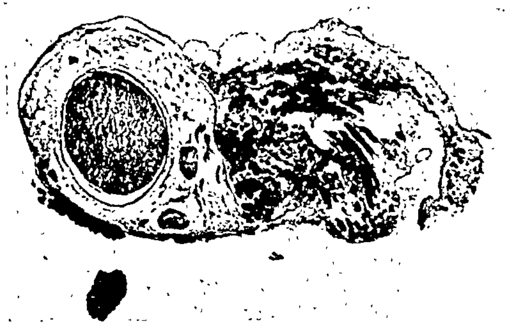

PLATE 1

PLATE 2

FIG. 2. VITAMIN A EXPERIMENTS

Results. Group 1 (A-deficient tryparsamide treated) (table 2). One animal in this group died after five weeks on the deficient diet. Death was apparently due to an intercurrent infection. The animal had been growing normally and showed no clinical signs of vitamin A deficiency.

The other four animals all developed frank signs of vitamin A deficiency during the ninth week on the diet, four weeks after tryparsamide had been started. Their weights, which had been showing a steady increase up to this point, stopped increasing and remained essentially unchanged until the end of the experiment. They developed sanguinous nasal discharges, the eyelids became edematous and a purulent conjunctivitis associated with xerophthalmia appeared. In some of the animals this progressed to sloughing of the cornea. All the animals appeared very lethargic. Their hair was very long and coarse.

TABLE 2

Rats on altered vitamin A diets

GROUP	DIET	DRUG	CLINICAL EVIDENCE OF VITAMIN DEFICIENCY	OPTIC NERVE DEGENERATION (HISTOLOGIC)
1	A-deficient	Tryparsamide 0.1 gm. per kilo for 10 weeks	Present	Advanced
2	A-deficient	None	Present	Extremely advanced
3	"Excess—A"	Tryparsamide 0.1 gm. per kilo for 10 weeks	None	None
4	"Suboptimal A"	Tryparsamide 0.1 gm. per kilo for 10 weeks	None	Moderate
5	"Optimal A"	Tryparsamide 0.1 gm. per kilo for 10 weeks	None	None

All of these signs increased in intensity during the succeeding six weeks. During the last three weeks (11–14 weeks on the deficient diet) the animals were all blind as a result of the xerophthalmia and they developed a staggering gait, apparently as a result of the deficiency. One animal died during the tenth week, the remaining three survived until sacrificed at the end of the fourteenth week, although they were obviously dying at that time.

Group 2 (A-deficient diet—no tryparsamide). The animals in this group developed signs of deficiency exactly similar to those described for Group 1, after eight weeks on the deficient diet. Although there was no qualitative difference in the clinical picture presented by the two groups, the rats in this group seemed sicker. Four of the five died, presumably as a result of A-deficiency, eleven to thirteen weeks after starting the diet and only one survived to be sacrificed at the end of the fourteenth week.

Group 4 (suboptimal A + tryparsamide). None of the animals in this group

showed any signs of vitamin A deficiency during life and the three survivors until the end of the experiment showed growth curves similar to the controls.

Two of the rats in this group died, apparently of an intercurrent infection, during the sixth week on the partially deficient diet. One of these animals had had only one, and the other only two injections of tryparsamide before death.

All the animals in *Groups 3* ("excess vitamin A" plus tryparsamide) and *5* ("optimal A" plus tryparsamide) showed normal growth curves and no evidence of any abnormality during the period of the experiment. Although conjunctivitis has been noted in rats on excessively high vitamin A intakes (37), the animals in Group 3 showed normal eyes throughout the entire period.

Histologic study. Sections of the optic nerves of the ten animals in Groups 1 and 2 (completely A deficient) all showed definite evidence of degneration. The picture was similar to that seen in the B-deficient animals. The nerves presented a loose edematous appearance. The nerve tissue seemed granular and the axones were poorly defined in cross section. In the tangential or longitudinal sections the axonal fibers showed marked variations in thickness, were beaded and fragmented. The same increase in number and size of the vacuoles seen in the degenerated nerves of the vitamin B experiment was also present here. These were considered to be presumptive evidence of increased ingestion of debris by the oligodendria cells.

Although the degenerative changes were qualitatively similar in the nerves of all the animals in these two groups, the most extensive changes were found in the sections from the animals in Group 2. These animals had *not* received tryparsamide.

In Group 4 ("suboptimal A" plus tryparsamide) the same type of degenerative change was seen but the extent was less marked than in either group of completely deficient animals. The sections from rats 1 and 5 of this group are of interest because of the fact that they had received so little tryparsamide (1 and 2 injections respectively). Despite this fact, degenerative changes were present which were indistinguishable from those found in the other members of this group.

Sections from the nerves of the Group 3 animals ("excess A plus tryparsamide") showed no degeneration and were entirely normal save for a very slight amount of gliosis in one animal.

In Group 5 ("optimal A" no tryparsamide), the sections from 4 of the 5 rats were entirely normal. In the remaining animal on one section only, there was very slight evidence of degeneration of the nerve with fragmentation of a few axones. Many other sections of this nerve were entirely normal (table 2).

Discussion. Examination of this material shows that rats subsisting on a synthetic diet completely deficient in vitamin A develop degeneration of the optic nerve, as had been shown by Lee and Sure (16). In contrast to the experiments with the B-complex, the changes with this A-deficient diet did not seem to be intensified by the administration of tryparsamide. In fact the clinical course seemed somewhat milder and the nerve degeneration less pronounced in the tryparsamide treated animals.

It is probable that this relatively beneficial effect of the tryparsamide is more

apparent than real, both because the number of animals involved is small, and because the degenerative changes in both groups are so extensive that exact measurement between them is difficult. It is probably more accurate to conclude that no demonstrable adverse effect of the tryparsamide was shown.

The results in Group 4 are extremely interesting and suggestive. All five animals receiving inadequate amounts of vitamin A plus tryparsamide showed degenerative changes in the optic nerves although there were no ocular or other clinical signs of vitamin A deficiency. The changes were present in two animals (1 and 5) *after one and two injections of tryparsamide respectively, on the* 36th and 38th days after being placed on the diet. Unfortunately no exactly comparable control data are available for this group but it would seem either that optic nerve degeneration may develop much earlier than the ocular and other clinical manifestations of A deficiency in rats, or that tryparsamide can damage the optic nerve in rats maintained on a low, but not completely deficient vitamin A intake. Experiments with larger numbers of animals are in progress to attempt to evaluate the relative importance of the two factors.

GENERAL SUMMARY AND CONCLUSIONS

1. Deficiencies of vitamin A and of several components of the B-complex were produced in rats. Certain groups of these deficient animals as well as normal rats were treated with tryparsamide.

2. Rats maintained on synthetic diets lacking only the complete vitamin-B complex showed degeneration of the optic nerve. This degeneration was intensified by the concurrent administration of tryparsamide.

3. Rats maintained on the same synthetic diet supplemented either with brewer's yeast or with all the available crystalline B-vitamins together (thiamin hydrochloride, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate and choline chloride) showed no degeneration of the optic nerve, whether or not they had received tryparsamide.

4. Rats maintained on diets which were partially deficient in pantothenic acid failed to show any degeneration of the optic nerves, whether or not they had received tryparsamide.

5. The deficiency responsible for the nerve degeneration in completely B-deficient rats is attributable to one or more of the known crystalline members of the group. It is not pantothenic acid alone.

6. Rats maintained on synthetic diets completely free of vitamin A developed marked degeneration of the optic nerves.

7. There was no evidence that the administration of tryparsamide intensified this process. The changes in the tryparsamide treated animals were, if anything, somewhat less than in the untreated animals, but this effect is probably only an apparent one as the changes were extensive in both groups.

8. Rats maintained on diets poor in vitamin A which were treated with tryparsamide, showed degenerative changes in the optic nerves, although they showed no clinical signs of A deficiency.

9. The degenerative changes in the low vitamin A group were of much less extent than in the completely deficient animals, either treated or untreated.

10. Rats maintained on synthetic diets supplemented by adequate amounts of B-complex (yeast) and vitamin A (cod liver oil) developed no degeneration of the optic nerve, whether or not they were treated with tryparsamide.

Aided by a grant from Merck and Company and the Barbara Henry Research Fund.
We wish to express our thanks to Mrs. Ralph Buchdahl for technical assistance, and to Miss Anna Mary McDowell who very kindly prepared the sections.

Vitamin-free casein, Labco brand was obtained from the Borden Company; dextrose used was the Corn Products Company's Cerelose; and the U.S.P. salt mixture xl was the number 1 or Osborne and Mendel mixture.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

III. THE CORRELATION OF PLASMA THIAMIN CONTENT WITH RESISTANCE TO SHOCK IN DOGS

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It has been shown in previous communications (1) (2) that the administration of thiamin to dogs in which shock has been induced by hemorrhage results, in many instances, in a prolongation of survival time and in a return to normal of the elevated keto acid, blood sugar, and blood lactic acid levels which occur in shock. Since dogs show marked variability in resistance to shock and response to treatment with thiamin, studies were made to determine whether or not any relationship exists between the amount of plasma thiamin and resistance to shock.

METHODS. Thirty-seven dogs were used as experimental animals. They were fed a standard diet of one pound of "Red Heart" dog food daily for one to two weeks before being used or placed on any special diet.

The animals were anesthetized with an initial dose of 32 mgm/kg. of Sodium Pentobarbital (Veterinary Nembutal Solution, Abbott), administered intravenously. Small amounts of the solution were given during the experiment as needed. All dogs were allowed to lie quietly under anesthesia for one hour before any samples were drawn or any bleedings were made.

Shock was produced by arterial bleedings at thirty minute intervals in the following amounts: 1.0% of body weight, 1.0%, 0.5%, 0.5%, 0.25%, and all further bleedings of 0.25% until the blood pressure remained within the range of 45–60 mm. Hg for thirty minutes. Any therapy used was applied after this thirty minute period. The blood pressure was recorded by an Anderson (13) glass capsule manometer connected to the cannulated left femoral artery. The animals were bled and blood samples for determinations were drawn from the cannulated right femoral artery. The right femoral vein was exposed for injections. Three to five blood samples of 11.0 cc. each were drawn for analyses. Plasma thiamin was determined by the method of Schultz, Atkin, and Frey (3), plasma specific gravity by the falling drop method, and hematocrit by the Wintrobe method.

An effort was made to vary the thiamin content (as measured by plasma thiamin determination) in several ways. The standard diet of the dogs, as above stated, was one pound of "Red Heart" dog food daily. This diet was found to be adequate in thiamin but some of the animals were given 1.0 mgm. thiamin daily by mouth in addition to the above. Other dogs were fortified with subcutaneous injections of thiamin solution for a few days before the experiment. A low plasma thiamin level was produced by feeding a synthetic diet of casein, sucrose, cottonseed oil, and agar, with cod liver oil and autoclaved Brewer's yeast, as used by Goodsell (12). The reduction of plasma thiamin produced by this diet was variable, and even after one month some of these animals still showed relatively high plasma thiamin levels. A few dogs were given large amounts of glucose by mouth with subcutaneous insulin. It was found that this procedure reduced plasma thiamin to low levels in three to four days. Three dogs on the thiamin-free diet were given a 1.0 mgm. tablet of thiamin daily by mouth. These dogs showed relatively high plasma thiamin levels and behaved as did those animals fed on the regular and thiamin fortified diets.

The data obtained from this series of dogs are summarized in table 1.

Discussion. Early in these experiments it was noticed that most of the dogs low in thiamin showed a blood pressure response to repeated bleedings distinctly different from that seen in the high thiamin animals. After each bleeding the blood pressure of the high thiamin dog immediately tended to rise to its former level and did not remain below 60 mm. Hg until the animal had been bled large amounts and was moribund. Death usually occurred suddenly, shortly after the last bleeding, in the high thiamin dogs. Twelve out of thirteen high thiamin dogs had an average survival time of thirty-four minutes after the last bleeding. The remaining animal lived seven hours.

The blood pressure response of the low thiamin dogs to the early bleedings (first two to four) was similar to that of the high thiamin animals, but the thiamin-poor dogs showed a rapid, spontaneous drop in blood pressure a few minutes after the last of two to four bleedings, the blood pressure then remaining between 45 and 60 mm. Hg for an average of 2.8 hours, after which the animals died. The blood pressure responses of the high thiamin dogs we have designated Type 1, and those of the low thiamin animals, Type 2. Several tracings of these types are shown in figures 1 and 2, respectively.

An intermediate type (Type 3) is occasionally seen, in which the blood pressure is markedly lowered after a few bleedings, similarly to Type 2, but in these cases the blood pressure gradually rises above 60 mm. Hg, before thirty minutes have elapsed, necessitating relatively large amounts of bleeding to produce the further drop of a few millimeters necessary to fulfill our criterion of shock. The plasma thiamin values of these animals are usually slightly below the average. Figure 3 shows tracings from such animals.

The curves of the plasma specific gravity and hematocrit determinations have been classified in Table 1 in the following manner:

1 = dilution
2 = dilution followed by concentration
3 = concentration
4 = concentration followed by dilution

In table 2 it will be seen that the dogs high in thiamin (4 μgm./100 cc. plasma and above) required 45.2% more bleeding for the induction of shock than did the low thiamin dogs, (0–2 μgm./100 cc.). Whereas 77.8% of the low thiamin animals went into shock with bleeding of less than 4.0% of body weight, none of the high thiamin dogs went into shock with this amount of bleeding, and only 43.8% of the high thiamin animals went into shock with less than 5.0% of body weight in blood withdrawn. The probability of chance occurrence of this difference is 7.5×10^{-5}.

All of the dogs were autopsied save 6 which recovered after treatment, and one other. The incidence of intestinal hemorrhage in these animals is shown in table 3. Six out of 7 dogs examined in the low thiamin group showed intestinal hemorrhage whereas none of 15 animals in the high thiamin group showed evidence of intestinal bleeding. The low thiamin dogs which were treated and

TABLE 1

DATE	WEIGHT	DIET OR PREPARATION	AMT. OF BLEEDING IN % BODY WEIGHT	INITIAL PLASMA THIAMIN IN γ/100 CC.	MIDDLE PLASMA THIAMIN IN γ/100 CC.	TERMINAL PLASMA THIAMIN IN γ/100 CC.	BLOOD PRESSURE TYPE	DROP IN PLASMA PROTEINS	CURVE OF PLASMA SPEC. GR.	CURVE OF HEMATOCRIT	TREATMENT	FATE OF ANIMAL
	kg.							gms. %				
7/15	7.26	20 gm./kg. sugar and 10 units insulin/kg.	2.7	0.4	4.5	20.0	2	0.17	1	3	Thiamin 5 mgm./kg. IV	Died 1 hr. later after transient rise in B.P. Marked intestinal bleeding
7/10	7.54	20 gm /kg. sugar and 10 units insulin/kg.	2.8	0.95	0.56		2	0.21	2	3	Thiamin 5 mgm./kg. IV	Improved and was repaired. Died during night. Marked intestinal bleeding
7/7	13.1	Low B_1 diet 1 month	4.45	1.0	0.6		2	0.51	1	3	Thiamin 5 mgm./kg. IV	Improved temporarily. Died 2 hrs. later. Marked intestinal bleeding
7/14	5.85	Low B_1 diet 1 month	3.3	1.05			2	0.85	1	1	Thiamin 5 mgm./kg. IV	B.P. rose after treatment. Died suddenly 1 hr. later. Autopsy: Bleeding in duodenum. Large hemopericardium
7/21	7.0	Low B_1 diet 3 weeks	2.3	1.3	1.39		2	0.58	2	4	Thiamin 5 mgm./kg. IV	Repaired, recovered completely
7/2	6.45	Low B_1 diet 3 weeks	3.5	1.4	3.4		2	0.48	1	3	Thiamin 5 mgm./kg. IV	Repaired, recovered completely
7/7	4.35	Low B_1 diet 4 weeks	4.5	1.8	2.55	3.05	3	0.61	1	4	Thiamin 5 mgm./kg. IV	Died 3.5 hrs. later. Marked duodenal and subendocardial hemorrhage
7/2	4.0	Low B_1 diet 3 weeks	3.0	1.9	4.8		2	0.57	2	3	None	Died 4 hrs. later. Marked duodenal hemorrhage
6/16	7.5	Low B_1 diet 4 weeks	3.2	2.0	0.9	1.15	3	0.71	2	1	Killed	No intestinal hemorrhage
5/26	8.7	Low B_1 diet 4 weeks	2.7	2.2	0.85		2	0.58	2	2	Thiamin 3 mgm./kg. IV	Repaired. Recovered completely
5/25	6.7	Low B_1 diet 4 weeks	2.75	2.2	2.9	3.4	2	1.14	1	4	None	Died 3.5 hrs. later. Marked hemorrhage in gut
6/29	11.5	Regular diet	5.05	2.2	1.05	3.9	1	0.54	1	3	Saline 20 cc./kg. IV	Died 3 hrs. later. Moderate congestion of gut. Slight duodenal hemorrhage
7/27	12.0	Low B_1 diet and 1 mg. thiamin daily for 2 weeks	5.05	2.45	0	2.45	1	0.91	1	4	None	Died 45 min. later. No hemorrhage. Slight duodenal congestion
6/16	5.95	Low B_1 diet 4 weeks	5.0	2.6	0.9	1.65	1(3?)	0.57	2	3	None	Died 3 hrs. later. No intestinal hemorrhage
7/14	7.2	20 gm./kg. sugar and 10 units insulin/kg. for 4 days	3.3	2.66			2	0.71	1	3	Thiamin 5 mgm./kg. IV	Repaired. Recovered completely
7/22	7.1	Low B_1 diet 3 weeks	3.8	3.0	3.78		2	0.07	1	3	Thiamin 5 mgm./kg. IV	Repaired. Recovered completely
5/22	6.7	Reg. diet and 1 mgm. thiamin/day 10 days	5.75	3.3	3.2	3.3	1	0.90	1	4	None	Died 30 min. later. No hemorrhage in gut

7/8	8.9	Reg diet 2 weeks	4.3	3 33	3.44		1	0.67	1	1	Plasma and thiamin	Died immediately after plasma infusion begun. Probably due to transfusion reaction. Plasma from donor dog No hemorrhage in gut. Few small hemorrhages in endocardium of left ventricle
7/22	8.6	Low B_1 diet 3 weeks	3.4	3.39	5.33		2	0 65	2	3	Thiamin 5 mgm /kg. IV	Died 1 5 hrs. later after transient rise in B.P. Marked hemorrhage in gut
7/21	12 8	Low B_1 diet 3 weeks	2.75	3.5	2.0	17.0+	2	0.44	2	4	Thiamin 5 mgm./kg. IV	Repaired. Recovered completely
6/30	9.75	Reg. diet 2 weeks	3.9	3.6	3.3	4.6	1	1.11	1	3	Plasma and thiamin	Died one day later Evidence of previous intraintestinal hemorrhage
5/20	9.2	Low B_1 diet 3 weeks	5 6	4.2	2 2	3.65	1	1.25	1	4	None	Killed 2 hrs. later. No bleeding in intestine
7/15	8.58	Low B_1 diet 3 weeks	4.45	4.3	4.0	12.6	1	0.65	1	3	Thiamin 5 mgm /kg. IV	Died 15 min. later. No bleeding in intestine
5/27	9.35	Reg. diet and 1 mgm. thiamin daily 15 days	5 0	4.5	7.2	9.0	1	0.69	2	2	None	Died 5 minutes after last bleeding. No hemorrhage in intestine
5/23	7.13	Reg. diet and 1 mgm. thiamin daily 15 days	4.75	4.7	4.4	5.8	1	1.22	1	4	None	Died 15 minutes after last bleeding No intestinal hemorrhage
5/6	12 1	Reg diet and 1 mgm thiamin daily 15 days	5.75	5.2	4.3	4.4	1	0.83	1	3	None	Killed 40 minutes after last bleeding. No intestinal hemorrhage
7/8	9.3	Reg diet 2 weeks	5 5	5.78	5 12	8.56	1	1.26	1	1	Plasma	Died immediately after plasma begun from donor dog. Probably transfusion reaction No intestinal hemorrhage
7/24	6.9	Low B_1 diet and 1 mgm. thiamin daily 2 weeks	5 25	6 0	24 9	3 45	1	0.95	1	4	None	Died 20 minutes after last bleeding No hemorrhage in intestine
7/27	9.15	Low B_1 diet and 1 mgm thiamin daily 2 weeks	5 5	6 34	0.80	7.78	1	0.72	1	4	None	Died 1 hr after 30 min. of shock. No intestinal bleeding
5/5	12 6 obese	Reg diet and 1 mgm thiamin daily 2 weeks	4 3	6 5	5.7	7 4	1	0.97	2	1	None	Died 30 minutes after last bleeding No intestinal hemorrhage
6/29	10.95	Reg diet 2 weeks	4 65	8.2	7.8	2.4	1	0.63	1	3	Saline and thiamin	Died 7 hrs later Autopsy not done
6/23	8.25	Low B_1 diet 4 weeks	4 35	9 0	6 7	4.5	1	0.81	1	1	None	Died 15 minutes after last bleeding. No hemorrhage in gut
6/22	4 9	Low B_1 diet 4 weeks	4.25	10 9	9 0		1	0.75	1	1	Thiamin 5 mgm /kg. IV	Died 1 hr after thiamin. No hemorrhage in intestine
6/23	14.65	Reg diet and 2 mgm thiamin/day for 3 days 100 mgm. thiamin IM before exp	4 65	22.6	17 8		1	0.67	1	1	None	Died 1 hr later. No hemorrhage in intestine
6/22	13.7	Reg. diet and 2 mgm thiamin/day for 3 days 100 mgm thiamin IM before exp.	5 75	29.8	30 2	35.6	1	0.65	1	1	None	Died 40 minutes after last bleeding No intestinal hemorrhage
6/15	9.3	Reg. diet and 5 mgm. thiamin sub cut. for 3 days	5.25	very high	very high	very high	1	0.89	2	1	Thiamin 5 mgm /kg. IV	Died 30 minutes after last bleeding. No intestinal hemorrhage
6/15	10.8	Reg diet and 5 mgm thiamin sub cut for 3 days	5 4	very high	very high	rose	1	0.86	2	1	None	Died 30 minutes after last bleeding. No intestinal hemorrhage

recovered passed tarry feces for a day afterward, suggesting that they also bled into the gut. This decrease in intestinal bleeding as the thiamin content increases is parallel with the decreasing frequency of hemoconcentration in high thiamin animals. This difference is also statistically significant ($p = 9.4 \times 10^{-5}$).

Some of these dogs were given intravenous thiamin solution (5.0 mgm./kgm.) as therapy after the thirty minute period of shock. It has been our experience that thiamin alone is not very efficacious in animals bled over 4.5% of their body weight, probably because of the large depletion of circulating blood volume.

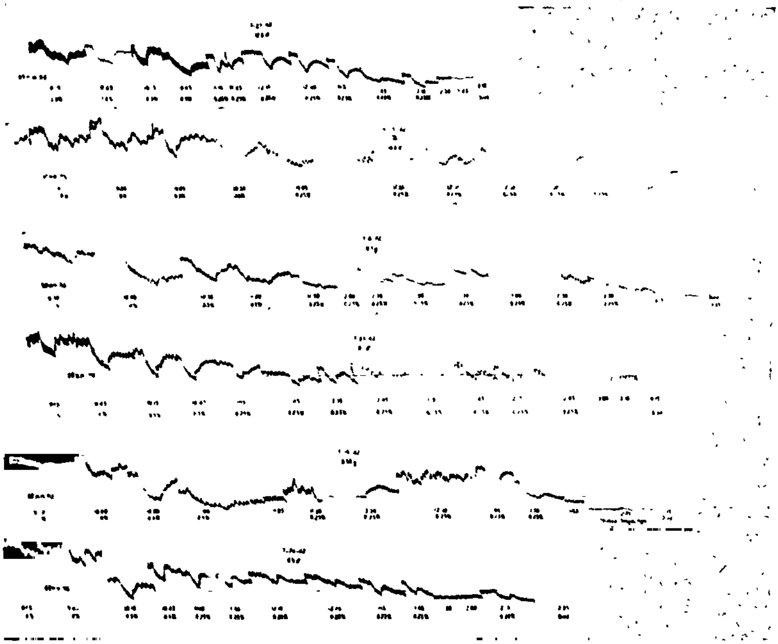

FIG. 1. BLOOD PRESSURE TRACINGS OF HIGH THIAMIN DOGS (TYPE 1)

As a consequence only three of the high thiamin dogs were given thiamin as treatment. As expected, they all died, since they had already withstood their maximum capacity for hemorrhage before developing hypotension. One would suppose that the low thiamin animals might show the most striking response to thiamin therapy, but it may be seen in Table 3 that this is not the case, probably because of the profuse intestinal bleeding in these cases. The maximum response to thiamin (80.0% complete recoveries) is to be seen in the intermediate group. One should note that these animals recovered completely, without any replacement of blood volume, or other therapy designed to remedy a possible tissue anoxia, with the exception of being allowed to drink water after

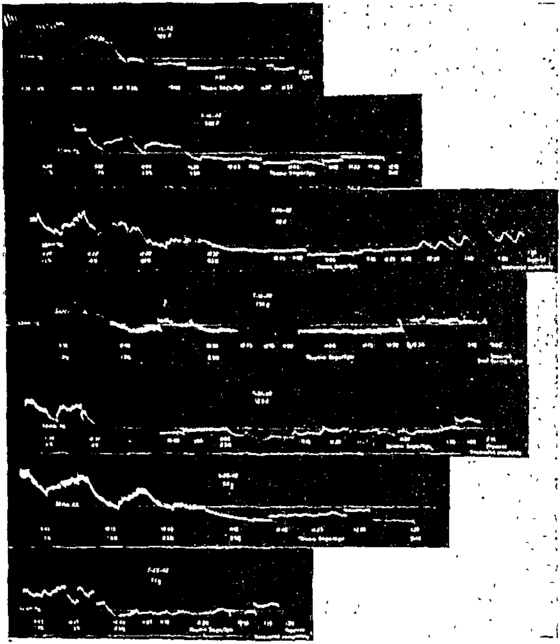

FIG. 2. BLOOD PRESSURE TRACINGS OF LOW THIAMIN DOGS (TYPE 2)

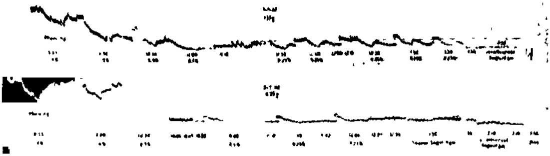

FIG. 3. BLOOD PRESSURE TRACINGS OF INTERMEDIATE DOGS (TYPE 3)

awakening from the anesthetic five to six hours later. The rise in blood pressure following thiamin administration to the low thiamin animals may be seen in figure 2.

In early experiments, done in an effort to find an explanation for the variability of animals in resistance to shock and in response to thiamin therapy, cocarboxylase[1] was given to a few dogs, on the basis that barbiturate anesthetics

TABLE 2

Amount of bleeding required for the induction of shock, correlated with initial thiamin levels

AMOUNT OF BLEEDING IN PER CENT OF BODY WEIGHT	LOW THIAMIN 0–2 γ/100 cc.	INTERMEDIATE THIAMIN 2–4 γ/100 cc.	HIGH THIAMIN 4 γ/100 cc. AND ABOVE
Less than 3%	33% (3/9)	25% (3/12)	0% (0/16)
Less than 4%	77.8% (7/9)	58% (7/12)	0% (0/16)
Less than 5%	100.0% (9/9)	66% (8/12)	43.8% (7/16)
Less than 6%	100.0% (9/9)	100% (12/12)	100.0% (16/16)
Average..........	3.25%	3.98%	4.72%

TABLE 3

Correlation of plasma thiamin levels with type of blood pressure, hemoconcentration, intestinal hemorrhage, and recovery after thiamin therapy

	LOW THIAMIN 0–2γ/100 cc.	INTERMEDIATE THIAMIN 2–4γ/100 cc.	HIGH THIAMIN 4γ/100 cc. AND ABOVE
Animals showing Type 2 blood pressure curve............	77.8% (7/9)	50.0% (6/12)	0% (0/16)
Animals showing hemoconcentration in plasma specific gravity curve...	45% (4/9)	33.3% (4/12)	25% (4/16)
Animals showing hemoconcentration by hematocrit.. ..	55.6% (5/9)	58.3% (7/12)	25% (4/16)
Average fall in plasma protein concentration............	0.52 gm./100 cc.	0.69 gm./100 cc.	0.86 gm./100 cc.
Animals showing intestinal hemorrhage (of those autopsied).	85.8% (6/7)	50.0% (4/8)	0% (0/15)
Animals recovering after thiamin therapy.	28.6% (2/7)	80.0% (4/5)	0% (0/3)

may depress certain liver functions, and among them, the mechanism for phosphorylation of thiamin. In some of these dogs in shock, a marked, immediate, blood pressure rise followed the administration of cocarboxylase, whereas there is always a lag of fifteen to thirty minutes before an effect is seen after thiamin administration. The same variation existed, however, in these dogs in the amount of bleeding needed for the induction of shock, and the response to therapy, as was found in the animals given thiamin, and these experiments were

[1] Kindly supplied through the courtesy of Merck and Company.

discontinued when the present series was begun. In view of the instability and difficulty of preparation of cocarboxylase, and consequent expense, the use of cocarboxylase in the treatment of shock could at this time be considered more of theoretical than of practical importance.

In table 1 it will be seen that in most cases the plasma thiamin level rose as the animal went into shock. We are aware of two explanations which may apply to this phenomenon. First, the hemodilution following hemorrhage may be responsible for the abstraction of thiamin from the tissues into the circulating blood. If this were true, it would seem that those animals showing a terminal hemoconcentration should also show a terminal fall in plasma thiamin. This is seen not to be the case, since the last plasma thiamin value is usually the highest. Second, the possibility exists that the plasma thiamin increase may be due to a mobilization of thiamin in response to the increase in substrate, namely pyruvic and other keto-acids.

We have previously (2) discussed in brief the need for thiamin in shock. Epinephrine liberation following hemorrhage produces maximal hepatic glycogenolysis and consequent hyperglycemia. Thiamin, as cocarboxylase, is indispensable for the proper metabolism of keto acids produced in intermediary carbohydrate metabolism. The question immediately arises as to whether or not such intermediary compounds as pyruvic acid are toxic to the animal in shock. Lu (4) suggests that if pyruvate itself is not toxic, it may be converted into other bisulfite binding compounds (such as pyruvic aldehyde) which may be detrimental in avitaminosis B_1.

However, to explain the beneficial effect of thiamin in shock, the postulation of a toxic factor and its removal is not necessary, since many tissues (e.g. nervous tissue) burn carbohydrate predominantly, and simple blockage of this metabolic cycle may seriously impair the functions of the tissue, without any intoxication in the usual sense.

The increase in portal pressure, splanchnic congestion and intestinal bleeding of animals in shock has been noted by many observers, among them Moon (5), and Erlanger (6). Lamson (7), Erlanger (6), and Bainbridge and Trevan (8) have shown that the first two of these effects can be duplicated by injection of large quantities of epinephrine. Lamson has suggested that this phenomenon may be caused by a constriction of the hepatic veins, whereas Bainbridge and Trevan state that the probable cause of the obstruction to portal drainage is a swelling of the hepatic polygonal cells. If Lamson's explanation be true, the work of Minz (9) and Glick (10), showing that thiamin intensifies the action of acetyl choline, may apply here, that is, if one assumes that acetyl choline may have a reciprocal action to that of epinephrine on the hepatic veins. The explanation of Bainbridge and Trevan is supported by the pathological observations of Nanta (11), who found that marked cellular damage occurred in the livers of fifteen humans dying after shock. Moon (5) also describes extensive hepatic parenchymatous degeneration. If these cells do swell as glycogenolysis occurs and cause intrahepatic obstruction to blood flow, then thiamin may be serving to support their metabolism so as to prevent or remedy these changes.

Recently Fitzgerald and Webster (14) have stated the possibility that barbiturates may cause a lowering of prothrombin, thus predisposing to hemorrhage. We wish to call attention to the fact that both our low thiamin dogs which showed marked intestinal hemorrhage, and the high thiamin dogs which did not, were anesthetized with pentobarbital-sodium.

It is recognized that these explanations are highly theoretical, but investigations are being carried out at present in this laboratory in an effort to clarify the problem.

We believe the picture seen in the thiamin-fortified dogs to be markedly different from that of the average and low thiamin dogs. One may not be able to go so far as to say that the dogs fortified with thiamin do not go into shock, but at least they develop hypotension after bleeding much later, after much more bleeding, and show much milder pathological changes at autopsy than do the low thiamin animals.

It seems highly desirable that thiamin be given clinical trial in the prevention and treatment of shock following hemorrhage. We believe that persons liable to be traumatized or wounded should be given adequate amounts of thiamin in their diet, that wounded individuals should receive an injection of thiamin solution as soon as possible after being seen by a physician, and that thiamin could be added to blood substitutes, such as plasma and saline-glucose solutions. The possibility of routine preoperative medication with thiamin should be considered.

Although we have not yet studied the effect of thiamin in surgical shock other than that induced by hemorrhage, it is known that disturbances of carbohydrate metabolism which require increased amounts of thiamin occur in these other types, and we believe that thiamin may possibly be found to have a beneficial effect in such conditions as traumatic shock, burns and severe dehydration.

CONCLUSIONS

1. Resistance to shock induced by hemorrhage in dogs anesthetized with pentobarbital-sodium is significantly greater in those animals having high plasma thiamin levels than in those showing low plasma thiamin values.
2. Dogs having high plasma thiamin values withstand more bleeding before developing severe hypotension than do animals having low plasma thiamin levels.
3. Dogs fortified with thiamin before bleeding show a constant tendency for their blood pressures to return to normal after hemorrhage, whereas low thiamin animals develop persistent hypotension early, after small amounts of hemorrhage.
4. The incidence of intestinal hemorrhage after bleeding is much greater in dogs low in thiamin than in animals having high plasma thiamin levels.

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THE EFFECT OF CHLOROFORM AND ETHER ON THE ACTIVITY OF CHOLINE ESTERASE

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There are some reports in the literature indicating that chloroform produces effects suggesting parasympathetic stimulation (1–3), while ether produces effects of parasympathetic depression (4–7). The underlying mechanism of these effects has not been established, and it is not known whether the apparent differences between the effects of chloroform and ether are qualitative or quantitative.

Bernheim and Bernheim (8) and Ettinger, Brown and Megill (9), using mainly biological methods, found that high concentrations of chloroform and ether inhibit choline esterase. Such concentrations of the two drugs are far greater than any concentrations achieved during anesthesia, and have been shown in animal experiments only to reduce the effect of acetylcholine or parasympathetic activity. The present study was undertaken with the view of throwing light on the effect of anesthetic concentrations of chloroform and ether on the activity of choline esterase. A chemical method was used to determine choline esterase activity because with biological methods no definite differentiation can be made between the inhibition of choline esterase and increased sensitivity of the effector cells.

METHODS. For this purpose the choline esterase activity of ground cat muscle was investigated. The method of Glick (10) was used, modified as follows: The choline esterase content of 200 grams of finely ground cat muscle was extracted with about 200 cc. of 154 mM NaCl solution for 4 hours at room temperature. The mixture was centrifuged and the supernatant fluid used as the solution of enzyme. The activity of solutions of the enzyme (controls or after chloroform or ether) was determined in the following manner: 1 cc. was incubated for 3 hours at 37°C. with 1.6 cc. of a 0.5% acetylcholine bromide solution. The latter was dissolved in a veronal buffer solution at pH 8, prepared by adding 7.15 cc. of 0.1 M sodium diethyl barbiturate to 2.85 cc. of 0.1 M HCl. The ether and chloroform were dissolved in the veronal buffer solution. The pH of the samples was adjusted to 8. The esterase activity was terminated at the end of 3 hours' incubation by the addition of an aqueous solution of 0.1% physostigmine salicylate. The choline esterase activity was then determined by the amount of acetic acid liberated during the 3-hour period of incubation. Glick accomplished this by titrating with a diluted solution of NaOH, using bromthymol blue as an indicator. In the present study it was found that comparison with a standard solution by means of a photoelectric colorimeter was more sensitive and exact. In the colorimetric comparison, the sample was boiled for 1 minute and filtered for the purpose of removing proteins which cause turbidity, after which 0.5 cc. of a 0.04% bromthymol blue solution was added to 5 cc. of the filtrate. The color reaction follows Lambert-Beer's law. The acid content of the samples was determined by a photoelectric colorimeter by comparison with a series of standard solutions consisting of the following: 1 cc. of 154 mM NaCl, 1.6 cc. of veronal buffer solution containing from $\frac{1\text{ N}}{200}$ to $\frac{3.5\text{ N}}{200}$ acetic acid, and 5 cc. of the

0.1% solution of physostigmine salicylate. To 5 cc. of the above mixture there was added 0.5 cc. of the 0.04% solution of bromthymol blue.

In several control experiments to test the reliability of the method using the photoelectric colorimeter, it was found that known concentrations of acetylcholine in water could be measured with an accuracy of ±5%.

One cc. of 1/200 N acetic acid is liberated from 1.13 mgm. acetylcholine bromide.

The amount of acetic acid liberated by the control samples tabulated in table 1 was determined by subtracting from the acid equivalent of incubated samples containing 1 cc. of muscle extract and 1.6 cc. of the acetylcholine buffer solution the acetic acid equivalent of the following control samples:

1) A sample containing 1 cc. of muscle extract and 1.6 cc. of the buffer solution without acetylcholine.

2) A sample containing 1.6 cc. buffer solution with acetylcholine and 1 cc. of water.

The amount of acetic acid liberated by the choline esterase in the presence of chloroform or ether was determined by subtracting from the acetic acid equivalent of the unknown solution the acetic acid equivalent of the following control samples:

1) A mixture of 1 cc. of muscle extract and 1 6 cc. of the buffer solution containing water, chloroform or ether in various concentrations.

2) A mixture similar to the foregoing, except that it contained acetylcholine without enzyme.

RESULTS. The effect of ether and chloroform on the choline esterase activity was tested for various concentrations of the two drugs: chloroform in concentrations of 0.01, 0.05 and 0.25 cc.%; ether in concentrations of 0.05, 0.5, 1.5 and 3.7 (approximately half-saturation) cc.%. These figures represent the concentrations of chloroform and ether in the samples at the start of incubation.

The results are summarized in table 1 and figure 1. It may be noted that both chloroform and ether exert an anti-choline esterase action. The intensity of the effect increases with the concentration of the drugs. Chloroform appears to be about 40 times as potent as ether. With concentrations of 0.25 cc. of chloroform % or 3.7 cc. of ether %, the choline esterase activity of the muscle is reduced by 25%. A statistical treatment of the data shows that these differences are significant and are not due to chance.

The concentration of the chloroform solutions used were 0.00125, 0.00627 and 0.03138 molar, and of ether 0.00479, 0.04792, 0.14386 and 0.35464 molar. A 15% inhibition is effected by a 0.00125 molar solution of chloroform and by a 0.04792 molar solution of ether. Thus the potency of chloroform is about 40 times that of ether. Physostigmine is much more effective than chloroform or ether, since a 0.01% solution of physostigmine nearly abolishes the choline esterase activity of muscle (about 80% reduction). A 0.01% solution of physostigmine contains 0.000364 gram-molecules. Interpolation of the data in figure 1 indicates that a 0.000364 molar solution of chloroform reduces the activity of the choline esterase by about 7%; therefore, chloroform is less than 10 times as effective as physostigmine. Physostigmine was used in the present work only in a concentration of 0.000364 molar. Bernheim and Bernheim found that a 0.000007 molar solution of physostigmine inhibits choline esterase nearly completely. Considering these data chloroform is about 500 times less potent an

COMPARATIVE PHYSIOLOGICAL ACTIONS OF SOME β-(IMIDAZOLYL-4-)ALKYLAMINES

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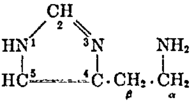
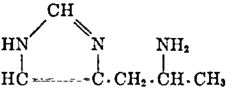
A considerable amount of work has been done on the effects of various substitutions in the β-(imidazolyl-*4*)ethylamine (histamine) molecule on its characteristic physiological activities (1). The *N*-alkyl derivatives, prepared by Garforth and Pyman (2) and later studied by Vartiainen (3), have proved to be the most active of all derivatives yet examined, but do not notably differ in their type of action. Of other type derivatives, the *5*-methyl compound described by Ewins (4) is among those of greatest activity, though it was reported to be but about as $\frac{1}{200}$ active a depressor as histamine, in the cat.

Histamine appears to be destroyed in the body by an aerobic enzyme system, first described by Best and McHenry (5), but further investigated as to its substrate specificity by Zeller (6), who renamed it diamine oxidase. Though this enzyme is distinct from amine oxidase in its substrate specificity, it appeared possible that similar structural changes in the substrates of these two enzymes might serve to prevent their oxidation. Blaschko, Richter and Schlossmann (7) had observed that the introduction of an α-methyl group in phenethylamine (β-phenylethylamine) or phenethanolamine (β-hydroxyphenethylamine) served to prevent the oxidation of the resultant compounds by amine oxidase. Correspondingly, in the present studies an α-methyl group was introduced into histamine and *5*-methyl histamine to determine whether such changes in their molecules would affect their oxidation by diamine oxidase. Further, it became of interest to discover whether their non-oxidizability by the diamine oxidase system resulted in any notable change in their physiological activity, as is the case when the phenethyl- and hydroxyphenethylamines are converted into the corresponding phenisopropylamines (see Alles (8, 9)).

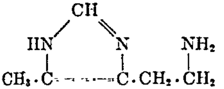
Experimental studies. The imidazolyalkylamines used in this work were synthesized and used in the form of their dihydrochlorides, with the exception of α-methylhistamine, which was better obtained as a solid in the form of its dihydrobromide. Histamine and α-methylhistamine were prepared from *1*, *4*-diaminobutanone-*2* and *1*, *4*-diaminopentanone-*2*, respectively, by their reaction with sodium thiocyanate, then oxidation of the resultant thiols, as described by Pyman (10) for the conversion of diaminoacetone into imidazolylmethylamine. *5*-Methylhistamine was prepared following Ewins (4), and its α-methyl derivative was prepared from aminoallylacetone following Sarasin (11). The basis of the nomenclature used to describe these compounds can be kept in mind by noting the following structural formulas:



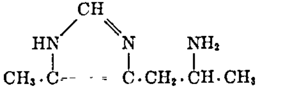
β-(Imidazolyl-4-)ethylamine
Histamine



β-(Imidazolyl-4-)isopropylamine
α-Methyl histamine



β-(5-Methylimidazolyl-4-)ethylamine
5-Methyl histamine



β-(5-Methylimidazolyl-4-)isopropylamine
α,5-Dimethyl histamine

Diamine oxidase substrate specificity. As source of the enzyme, hog kidney powder was prepared as described by McHenry and Gavin (12) and an extract made from 10 grams of the powder with 490 cc. M/15 phosphate buffer pH 7.2 by agitating for one hour at 38°. The filtered extract was made up to 500 cc., then divided into five 100 cc. portions. To each portion was added 2 cc. hexanol to decrease frothing and to keep the mixture sterile. One portion was run as a control while the others were made up to 0.0002 molal with histamine (*A*), 5-methylhistamine (*B*), α-methylhistamine (*C*), and α,5-dimethylhistamine (*D*), respectively. Oxygen was bubbled through sintered glass bubblers into each solution for 24 hours at 38°, and aliquot samples were removed at intervals for analysis of their ammonia content.

In such experiments the aeration method of estimating ammonia, as had been used by McHenry and Gavin (12), was first tried, but the precision obtained was not sufficient. By following the Conway and Byrne (13) ammonia distillation procedure, and titrating the evolved ammonia using a glass electrode submicrotitration procedure like that of Borsook and Dubnoff (14), the accuracy of analysis was improved sufficiently. While the oxidation of histamine and 5-methylhistamine proceded as shown in figure 1, the ammonia values observed in the samples containing their α-methyl derivatives were in no case significantly different from those of the controls.

Depressor effects in dogs. The effects upon arterial pressure following intravenous injection into dogs under ether or sodium pentobarbital anesthesia, or pithed, were compared. The three derivatives of histamine were notably less active in their depressor effects than histamine itself, the α- and 5-methyl derivatives each being about $\frac{1}{10}$ as active, and the α,5-dimethyl derivative only about $\frac{1}{1000}$ as active. In comparison with 10^{-8} mols/kg. of histamine, the effects of 10^{-6} mols/kg. of α-methylhistamine are notably much more prolonged. Whether this increased duration is to be ascribed to a greater stability of the compound in the body was not made clear, for 5-methylhistamine in a dosage of 10^{-6} mols/kg. also has a very considerable duration of action. The effects of 10^{-5} mols/kg. of α,5-dimethylhistamine are of comparable intensity, and also are very pro-

longed. The long-lasting effects of considerably active doses of the histamine derivatives precluded any more precise valuations of their relative activities by serial injections. In the minimal dosage range used, no considerable broncho-constrictor or other effect on respiration was noted.

Effects on isolated rabbit intestine. The sensitivity of intestinal strips often varies considerably in passing from jejunum to ileum to colon with respect to sensitivity to histamine. The jejunum commonly is markedly less sensitive to adrenaline and acetylcholine, and somewhat less sensitive to histamine, than is the ileum. The rectal colon may be somewhat less sensitive than the ileum to adrenaline and acetylcholine, but usually affords a much greater contraction to histamine. This variation in sensitivity is notably evident in figure 3, where in *A* are shown the successive responses to these several agents in different concen-

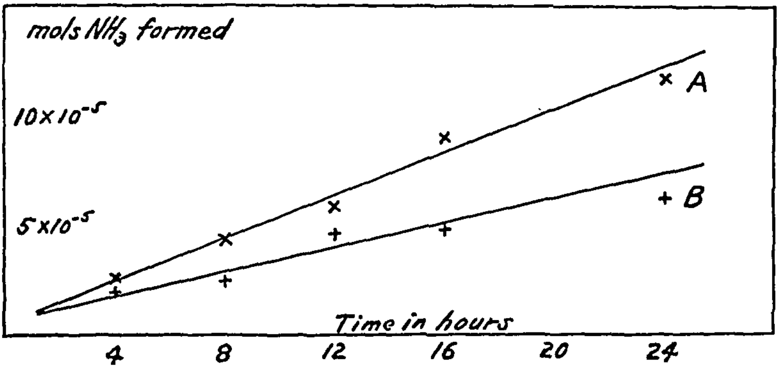


FIG. 1. Ammonia formation from 20 × 10^{-5} mols per 100 cc. of (*A*) histamine, (*B*) δ-methylhistamine, in a 2% hog kidney powder extract with M/15 phosphate buffer pH 7.2, on aeration at 38′. Under same conditions no significant ammonia formation from (*C*) α-methylhistamine or (*D*) α,δ-dimethylhistamine.

trations upon a single strip of jejunum, in *B* upon a single strip of ileum, and in *C* upon a single strip of colon, with all three strips taken from the same rabbit.

A 10^{-3} molal concentration of histamine is usually required to have a marked effect upon ileum, with the resultant contraction somewhat less than that from 10^{-6} molal acetylcholine. Further increase in the histamine concentration commonly causes less contraction, and with 10^{-2} molal only an inhibition of tone and movement usually results.

Only in minimally active concentrations do the methyl derivatives of histamine show contraction effects upon the ileum. They are inactive below about 5 × 10^{-3} molal, and at this concentration usually exhibit a slight contraction effect followed by some inhibition of tone and movement. The tendency is relatively marked to inhibit both tone and movement, and at 10^{-2} molal only this effect is notable.

One can conclude that the methyl derivatives are not greatly different from each other in their actions, and that they are less than $\frac{1}{5}$ as active as histamine in

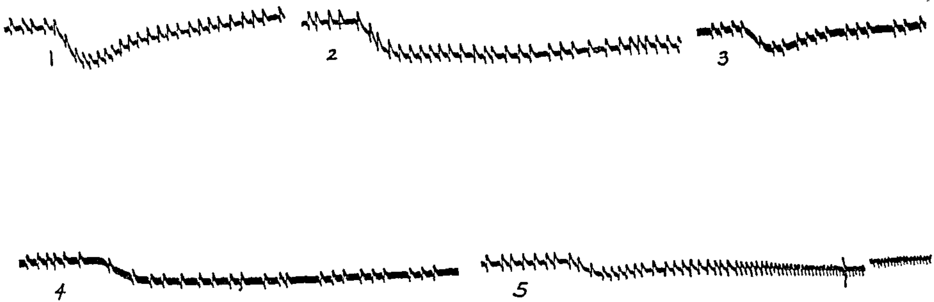


FIG. 2. DOG, ETHER. CAROTID ARTERIAL PRESSURE TRACINGS

1, 10^{-8} mols/kg. histamine; *2*, 10^{-6} mols/kg. α-methylhistamine; *3*, after 15 minutes, 10^{-8} mols/kg. histamine; *4*, 10^{-6} mols/kg. *5*-methylhistamine; *5*, 10^{-6} mols/kg. α,*5*-dimethylhistamine and 5 minutes after.

causing any contraction, but about equivalent in their depression of tone and movement with high concentrations. Antagonism by atropine, particularly of any contraction effect of histamine or its methyl derivatives, is notable with equimolal dosages, though the effects of atropine itself are inhibitory to some extent at 5×10^{-3} molal, and marked at 10^{-2} molal, which does not always permit a conclusion from each experimental trial.

Effects on isolated guinea-pig intestine. The sensitivity of intestinal strips, as with the rabbit, varies in passing from jejunum to colon, but while this may be particularly notable with adrenaline, the change with acetylcholine and histamine is much less. The guinea-pig intestine as a whole, however, is far more sensitive to histamine, and somewhat more sensitive to acetylcholine, than is rabbit intestine under the same conditions of testing. Whereas the effect of 10^{-3} molal

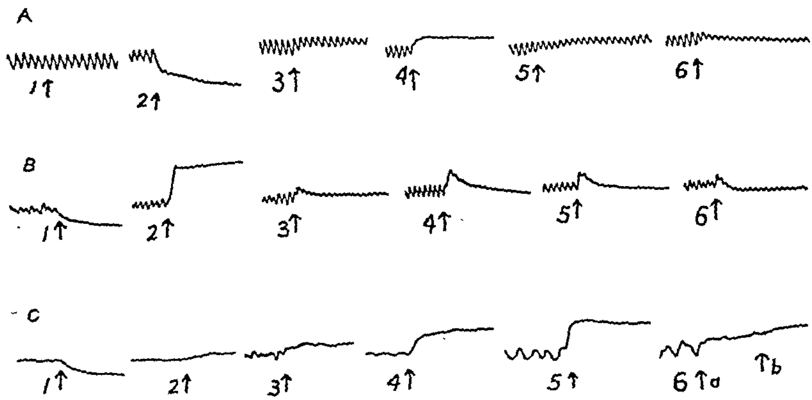


FIG. 3. ISOLATED RABBIT INTESTINAL STRIPS. CONCENTRATIONS IN MOLS/LITER
A, Jejunum: 1, 10^{-5} epinephrine; 2, 10^{-4} epinephrine; 3, 10^{-5} acetylcholine; 4, 10^{-4} acetylcholine; 5, 10^{-4} histamine; 6, 10^{-3} histamine. B, Ileum: 1, 10^{-6} epinephrine; 2, 10^{-6} acetylcholine; 3, 5×10^{-4} histamine; 4, 10^{-3} histamine; 5, 2×10^{-3} histamine; 6, 5×10^{-3} histamine. C, Rectal colon: 1, 10^{-6} epinephrine; 2, 10^{-6} acetylcholine; 3, 5×10^{-5} histamine; 4, 10^{-4} histamine; 5, 10^{-3} histamine; 6a, 10^{-3} atropine, then 6b, 10^{-3} histamine.

histamine on rabbit intestine may be somewhat less than with 10^{-6} molal acetylcholine, on guinea-pig intestine 10^{-7} molal of histamine or acetylcholine always caused marked and similar contraction effects, and on occasion even 10^{-8} molal histamine was active. In figure 4 are shown the successive responses of a single strip of guinea-pig ileum to acetylcholine, histamine and its derivatives of present interest.

Dependent on the particular preparation and the concentration level used for testing, 5-methylhistamine was from $\frac{1}{100}$–$\frac{1}{200}$, α-methyl histamine from $\frac{1}{150}$–$\frac{1}{300}$, and α,5-dimethylhistamine from $\frac{1}{300}$–$\frac{1}{600}$, as active as histamine on ileum strips of guinea-pig intestine.

Effects on isolated mouse intestine. Rarely have the effects of drugs on the smooth muscles of the mouse been investigated. Due to our finding of considerable differences in lethal toxicity in the guinea-pig and mouse with histamine

and its derivatives, it became of interest to determine if similar differences existed in the reactivity of smooth muscle of the intestine. It was found that concentrations of about 10^{-5} molal adrenaline or 10^{-6} molal acetylcholine were active in producing relaxation or constriction, respectively. Concentrations of about 10^{-3} molal of histamine and its methyl derivatives were required to show any effect, and this was only a slight decrease in tone and inhibition of movement. No differences were evident in the relative inhibitor activity of these compounds.

Acute lethal toxicity in mice. The intravenous lethal dose for histamine has been reported as from 250–300 mgm./kg, and by subcutaneous injection from 600–2000 mgm./kg, by various workers (1). In the present study, intraperitoneal injections were made into mice obtained from a single source and six to eight weeks old, ranging from 15–25 grams in weight. Lethal dosages resulted in marked depression, with convulsions and gasping for air immediately before

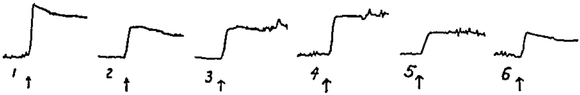


FIG. 4. ISOLATED GUINEA-PIG ILEUM STRIP. CONCENTRATIONS IN MOLS/LITER
1, 5 × 10^{-8} acetylcholine; 2, 5 × 10^{-8} histamine; 3, 10^{-5} δ-methylhistamine; 4, 2 × 10^{-5} α-methylhistamine; 5, 2 × 10^{-5} α,δ-dimethylhistamine; 6, 5 × 10^{-8} histamine.

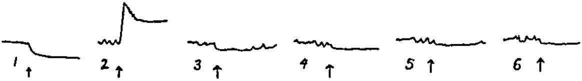


FIG. 5. ISOLATED MOUSE ILEUM. CONCENTRATIONS IN MOL/LITER
1, 10^{-5} epinephrine; 2, 10^{-6} acetylcholine; 3, 10^{-3} histamine, 4, 10^{-3} δ-methylhistamine; 5, 10^{-3} α-methylhistamine; 6, α,δ-dimethylhistamine.

death. Pilomotor response was notable, exophthalmos common, and autopsy revealed occasionally some hemorrhagic spots in the lungs. In all autopsies there seemed to be an excessive amount of fluid in the peritoneal cavity.

Acute lethal toxicity in guinea-pigs. As noted previously by others (see (1)), the relative toxicity of histamine for guinea-pigs is greater than for other animals. Death appears to result primarily from the marked bronchoconstriction produced and is due to asphyxia. For the present studies guinea-pigs weighing 400–600 grams were injected intraperitoneally. Death in all cases from histamine appeared to be due to the marked bronchoconstriction; inflated lungs were very notable at autopsy but there was no evidence of lung hemorrhage. With the methyl derivatives of histamine, depression and some difficulty with breathing, with gasping before death, were noted. Distention of lungs was not marked at autopsy, but dilation of the blood vessels of intestines, abdominal walls and viscera generally was notable. Death following histamine injection usually occurred within 15–30 minutes, while with the methyl derivatives it commonly

occurred only after a longer period and sometimes up to 5–8 hours. Whether or not the animal died seemed to depend more on its bodily reserves, and this factor may account for the variation in lethality with different animal groups.

TABLE 1

Lethal doses for half of mice receiving intraperitoneal injections

COMPOUND	DOSE	DOSE	LIVED	DIED	LD_{50}
	mM./kg.	*mgm./kg.*			*mM./kg.*
Histamine	6.0	1104	9	1	7.0
dihydrochloride	8.0	1472	3	7	
5-Methylhistamine	5.0	990	6	4	5.0
dihydrochloride	6.0	1188	1	9	
α-Methylhistamine	3.0	861	10	0	3.5
dihydrochloride	4.0	1148	3	7	
α,5-Dimethylhistamine	3.0	636	9	1	3.5
dihydrochloride	4.0	848	0	10	

TABLE 2

Lethal doses for half of guinea-pigs receiving intraperitoneal injections

COMPOUND	DOSE	DOSE	LIVED	DIED	LD_{50}
	mM./kg.	*mgm./kg.*			*mM./kg.*
Histamine	0.02	4	5	0	0.025
dihydrochloride	0.03	6	1	4	
5-Methylhistamine	0.8	158	3	2	
dihydrochloride	1.0	198	3	2	1.0
	1.5	297	0	5	
α-Methylhistamine	0.8	230	3	2	
dihydrobromide	1.0	287	3	2	1.0
	1.5	530	1	4	
α,5-Dimethylhistamine	0.8	170	5	0	
dihydrochloride	1.0	212	2	3	1.5
	1.5	318	5	0	
	2.0	424	1	4	

EXPERIMENTS IN MAN. The intradermal injection of histamine in sufficient concentrations results in the triple response reaction of Lewis (15). After considerable preliminary testing in the skin of two of the authors, simultaneous skin tests were run with the four compounds to determine their threshold concentrations for producing the skin reaction. The tests were made comparatively on both right and left forearms by pricking a test needle through a drop of solution of an ascending and descending dilution series, placed on the arm from wrist to

elbow. The thresholds were determined for each compound within about 100% variation. On both G. A. and M. S. the thresholds were: histamine, M/1000; 5-methylhistamine, M/20; α-methylhistamine, M/5; α-5-dimethylhistamine, M/20. The reactions with the solutions of the methyl derivatives of histamine appeared redder when fully developed, and lasted longer.

Histamine appears to be quite rapidly destroyed in the body, and doses of 100 mgm. administered orally to man were found to be inactive by Nathanson (16). The rate of destruction in man was established by Weiss, Robb and Ellis (17) as about 0.003 mgm. histamine per minute, and they found dosages as high as 200 to 500 mgm. of histamine diphosphate (65 to 165 mgm. histamine) given orally were without distinct effect on the circulation.

While it is not at all clear at present whether diamine oxidase oxidation in the body is a major mechanism for this rapid a destruction of histamine, it was of much interest to determine if α-methylhistamine was orally active, since it had been demonstrated that it was not oxidizable by diamine oxidase. After some preliminary trials, a series of dosages of 50, 100 and 200 mgm. of α-methylhistamine dihydrobromide were administered orally to G. A., and observations made on the circulation. The dosage was administered about two hours after a light morning meal by swallowing a half-glass of water containing the material in solution, and observations were continued for two hours while remaining at rest. No change in blood pressure, heart rate, the electrocardiogram, or skin temperatures of several parts of the body, occurred. No change in respiration or appearance of subjective symptoms indicating any peripheral or central nervous system changes were noted with any of the doses tried.

Discussion. The non-oxidizability of the α-methyl derivatives of histamine and 5-methylhistamine in the presence of diamine oxidase parallels the relations found with regard to amine oxidase substrate specificity. It would be of interest to determine if indeed these α-methyl derivatives might not only be non-oxidized by *diamine oxidase, but would act as inhibitors of the oxidation of histamine by* the enzyme. A similar situation was found by Blaschko (18, 19) with regard to the influence of α-methylphenethylamines on the action of amine oxidase. That diamine oxidase oxidation does play the limiting role in the rate of inactivation of injected histamine may, however, be seriously questioned. As shown by the data of figure 1 with enzyme in excess and substrate in a concentration of 2×10^{-3} molal, oxidation of half of this requires about 10 hours. In contrast to this, the data of Weiss, Robb and Ellis (17) show that the inactivation rate in the body is such that, with an injection rate of about 10^{-4} mgm. per kg. per minute of histamine diphosphate, the rate of inactivation equals the rate of injection. Such an equilibrium is at a concentration of about 10^{-3} mgm. per liter of blood, or 3×10^{-9} molal histamine. The enzymic oxidation rate should be $3 \times 10^{-9} / 2 \times 10^{-3}$ that of the experiment of figure 1, but the inactivation rate is actually almost comparable. It would seem that some other more rapid process, such as adsorption on the enzyme or receptive substances, is necessary to account for the relatively high rate of inactivation.

The pharmacological data presented indicate that for the various actions that

are characteristic and fairly specific for histamine as compared with other types of humoral agents, the activities of the methyl derivatives are all much decreased. The depressor effect in the dog, the contraction effect on guinea-pig intestine, and the acute toxicity in the guinea-pig resulting from bronchoconstriction, are examples of such actions in animals. With these reactions, 5-methylhistamine and α-methylhistamine are in all cases from $\frac{1}{50}$ to but about $\frac{1}{200}$ as active as histamine, and the α-5-dimethylhistamine usually somewhat less active in comparison. The triple response to intradermal injections in man also has a highly specific character for histamine, and here again histamine was much more active than the methyl derivatives presently studied.

SUMMARY

1. In dogs under ether or pentobarbital anesthesia, or pithed, histamine is an active depressor, and its α- and 5-methyl derivatives are about $\frac{1}{100}$ as active, while its α-5-dimethyl derivative is only about $\frac{1}{1000}$ as active.

2. On isolated rabbit or mouse intestinal strips, histamine and these methyl derivatives are all relatively inactive.

3. On guinea-pig intestinal strips, histamine exerts characteristic contraction effects, and the effects of its α- and 5-methyl derivatives are but $\frac{1}{100}-\frac{1}{300}$ as great, with the effects of its α-5-dimethyl derivative even less.

4. While histamine is somewhat less toxic for mice than these methyl derivatives, it is 40 or more times as toxic for guinea-pigs, apparently due to its marked bronchoconstrictor effects in this species.

5. In producing the triple response in the skin of man, histamine is also at least 50 times more active than the methyl derivatives studied.

6. Like histamine, α-methylhistamine was found to orally inactive in dosages as high as 200 mgm. of the dihydrobromide.

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TOXICITY OF TANNIC ACID

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During the past two years considerable interest has been focused on the treatment of burns. In spite of the introduction of a number of new techniques (1, 2) and preparations (3, 4), tannic acid still remains one of the most important forms of burn therapy. It is recognized that tannic acid has certain definite limitations and restrictions, particularly with respect to the treatment of burns of the extremities (5, 6). In addition, this agent appears to be harmful to the liver as reported recently by Wells (7).

It seemed important therefore to investigate the toxicity of tannic acid[1] in animals with respect to its effect when employed in conditions similar to those used in the clinic.

General course of investigation. These experiments were undertaken with the object of determining the toxic effects of tannic acid using experimental procedures which resemble as closely as possible the clinical conditions under which this drug is employed. For this purpose we applied tannic acid, in the form of an aqueous solution or as a dry powder, to surgically denuded areas in mice, rats and rabbits. In this manner tannic acid was applied to the deep subcutaneous tissue such as encountered after surgical debridement in severe burns. In view of the fact that we were determining the toxic rather than the therapeutic effect of tannic acid, experimentally burned animals were not used, since this would have introduced an additional, and not easily controllable variable.

To aid in determining the type and character of the toxic signs produced by tannic acid, a number of acute toxicity experiments were performed in which the drug was administered intravenously, subcutaneously or orally. In addition, kidney and liver function tests were performed.

Finally, since tannic acid eschars appear to undergo chemical and physical change during the first 24 hours after application, it seemed of interest to determine whether the products formed were more toxic than the parent substance. In connection with the toxicity of degradation products of tannic acid, the toxicity of gallic acid was studied in view of the fact that this compound is the main product formed on the hydrolysis of tannic acid.

Acute toxicity experiments. These were performed by administering tannic acid intravenously, subcutaneously or orally to mice and rats in the form of a 10% aqueous solution. Single doses of 5 to 100 mgm. per kilogram were given intravenously and doses of 50 to 4000 mgm. per kilogram were injected subcutaneously. Much larger doses (1–10 grams per kilogram) were given orally. Observations were made at frequent intervals during the first 5 days and thereafter once daily for 5 additional days. The results of these experiments are presented in table 1.

Shortly after intravenous or subcutaneous administration in mice, the respira-

[1] The tannic acid used in these experiments was Merck U.S.P. Tannic acid obtained from Chinese nut gall.

tion became slow and the temperature dropped significantly. Five to ten per cent of the mice developed paralysis of the hind limbs which persisted until death. The most striking finding appeared to be the effect upon the eyes. Within twenty-four hours following the intravenous or subcutaneous adminis-

TABLE 1

Toxicity of tannic acid in mice

DOSE	NUMBER OF MICE	NUMBER DEAD							TOTAL NUMBER DEAD
		Time in days							
		1	2	3	4	5	6	7	
				Oral administration					
gm./kgm.									
1.0	20	0	0	0	0	0	0	0	0
2.0	20	0	1	1	0	0	0	0	2
2.5	20	0	0	0	0	1	1	0	2
3.0	20	2	0	2	1	0	0	1	6
4.0	20	9	0	6	1	0	0	0	16
5.0	20	11	0	6	0	0	0	0	17
6.0	20	17	0	3					20
7.5	20	17	3						20
10.0	20	19	1						20
				Intravenous administration					
5	20	0	0	0	0	0	0	0	0
10	20	1	0	0	0	0	0	0	1
20	20	2	0	0	0	0	0	0	2
40	20	6	0	1	0	0	0	0	7
60	20	16	1	1	0	0	0	0	18
80	20	17	3						20
100	20	20							20
				Subcutaneous administration					
50	20	0	0	0	0	0	0	0	0
75	20	0	0	1	0	0	1	1	3
100	20	0	1	3	1	1	0	0	6
125	20	2	2	3	1	0	0	0	8
150	20	0	19	0	0	0	0	0	19
175	20	14	4	0	0	0	0	0	18
200	20	15	5						20
250	20	17	1	1	0	0	0	0	19
400	20	9	9	2					20

tration of doses which were not lethal, the cornea of many mice became cloudy and the contents of the entire eye turned dark-red. Histological examination showed this to be the result of hemorrhage into the eye. In such animals both the corneal and the pupillary reflex were absent and the animals appeared to be blind. Eventually most of these mice died. Subcutaneous injection of tannic

acid produced a small hard nodule, apparently due to the effect of tannic acid on tissue proteins.

Rats were much more resistant to tannic acid than mice. Doses of 3 to 4 times those administered to mice were required to produce toxic signs in rats. None of these showed hemorrhage into the eye even with large doses. An occasional rat showed evidence of hematuria following large doses subcutaneously. In both mice and rats, tannic acid or possibly its degradation products could be detected in the urine by adding salts of heavy metals to form a characteristic blue-black precipitate.

TOXICITY OF TANNIC ACID SUBJECTED TO AIR OXIDATION. Since tannic acid assumes a black appearance within 24 hours following its application to burned tissues, it seemed of importance to determine whether this effect was due to air-oxidation, and if the products resulting from this reaction were more toxic than the parent substance. It also seemed of interest to determine whether the toxic signs observed after the administration of tannic acid could be produced by gallic acid, the latter being the main product formed on hydrolysis of this drug.

Tannic acid was subjected to air oxidation by passing a current of air or oxygen through an aqueous solution for a 48 hour period. These solutions became somewhat darker in color, but did not assume the marked black appearance characteristic of the tannic acid applied to burns. There was no significant difference in the toxicity of tannic acid before and after the above treatment as determined by subcutaneous injection into mice. These results suggest therefore that the physical change observed in the application of tannic acid to denuded surfaces are not due solely to air oxidation, but may possibly be the result of a reaction between the drug and the tissues.

Toxicity experiments performed with gallic acid indicates that this compound is less toxic than tannic acid. Furthermore, none of the toxic signs produced by tannic acid in mice were observed following the administration of large doses of gallic acid. It seems unlikely therefore that the toxic effects produced by tannic acid in mice are due to gallic acid.

TOXICITY FOLLOWING THE APPLICATION OF TANNIC ACID TO DENUDED SURFACES. After the character of the signs of tannic acid poisoning had been established, we proceeded to study its effects following its application to denuded surfaces.

The procedure employed consisted in removing a definite area of skin from the backs of mice (2 sq. cm), rats (20 sq. cm.) or rabbits (108 sq. cm.) under ether anesthesia, and applying to the denuded surface tannic acid in the form of a thick paste or as a 20% aqueous solution. All animals were housed in individual cages throughout these experiments.

Three types of experiments were performed. In the first, a single application of tannic acid was made to denuded backs of animals. The second group received a single application of tannic acid, but in addition, the eschar was rewetted daily over a 10 day period to facilitate further absorption of the drug. Finally, a group of animals was treated daily for 10 days with tannic acid. In the latter experiment the 24 hour eschar was removed as completely as possible with warm sterile water before applying fresh tannic acid. In each of the 3 series the animals were observed daily over a 10 day period for toxic signs. For

each of the foregoing experiments an equal number of control animals were treated with saline instead of tannic acid. Animals dying during this interval were examined for possible bacterial infection by means of the usual bacteriological techniques. In addition the tissues of a large percentage of the animals were retained for histological study.[2]

TABLE 2

Toxicity of tannic acid in mice following its application to denuded surfaces

NUMBER OF MICE	SUBSTANCE	NUMBER SURVIVING ON DAYS				
		1	2	3	4	5
50	Tannic acid	47	25	7	0	0
50	Saline controls	50	50	50	50	50

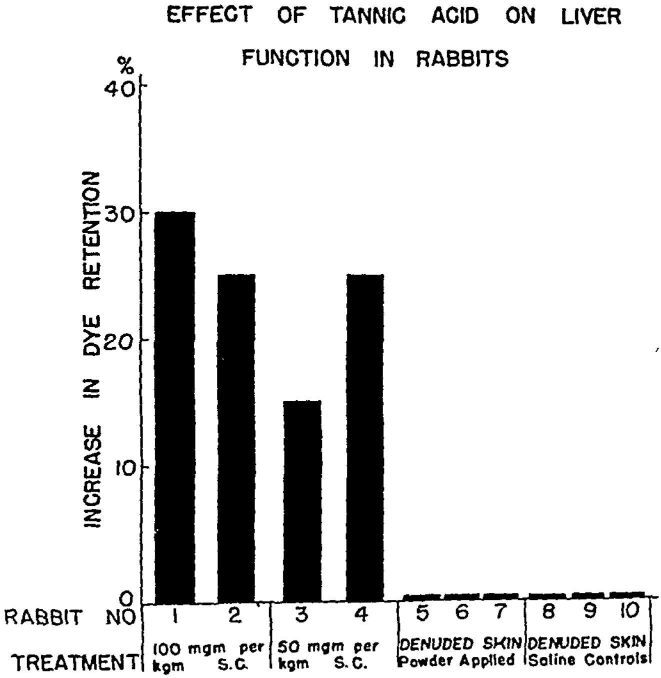

FIG. 1. EFFECT OF TANNIC ACID ON LIVER FUNCTION IN RABBITS WHEN INJECTED SUBCUTANEOUSLY OR APPLIED TO DENUDED SURFACES IN RABBITS. (BROMSULPHALEIN TEST)

From the results shown in table 2, it is apparent that single application of tannic acid in the form of a thick paste are lethal for mice. In general, the toxic signs produced were similar to those obtained by subcutaneous administration. The eyes of a number of mice became cloudy and hemorrhagic and

[2] These will be reported elsewhere by Dr. Henry Siegel.

the same characteristic paralysis of the hind limbs was observed. Tannic acid, or possibly its degradation products, could be demonstrated in the urine by chemical methods, which indicates absorption and excretion of the drug or its products. Control mice treated with saline remained normal. When single or repeated applications of tannic acid were applied to rats or rabbits no outward evidence of toxicity was observed. It will be recalled that rats were also more resistant than mice to tannic acid when injected parenterally. When the tannic acid eschar was rewetted, or when the drug was applied daily over a 10

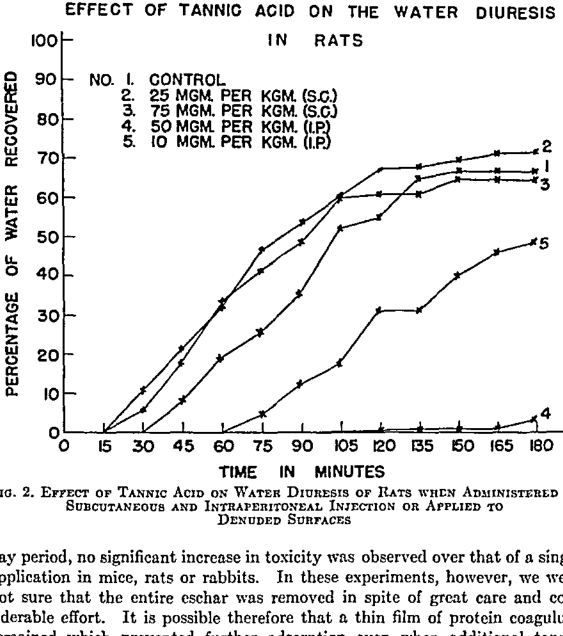

FIG. 2. EFFECT OF TANNIC ACID ON WATER DIURESIS OF RATS WHEN ADMINISTERED BY SUBCUTANEOUS AND INTRAPERITONEAL INJECTION OR APPLIED TO DENUDED SURFACES

day period, no significant increase in toxicity was observed over that of a single application in mice, rats or rabbits. In these experiments, however, we were not sure that the entire eschar was removed in spite of great care and considerable effort. It is possible therefore that a thin film of protein coagulum remained which prevented further adsorption even when additional tannic acid was applied.

EFFECT ON THE LIVER. The bromsulphalein test of Rosenthal (8) or its modification by Seeler and Kuna (9) for small animals were used to determine the effect of tannic acid on the liver function of rabbits and rats. After control

values had been established tannic acid in doses of 10 mgm. per kilogram was injected subcutaneously or applied to denuded surfaces in the form of a thick paste. Liver function tests were performed frequently over a two week period during which time the tannic acid administration was continued.

When injected subcutaneously, tannic acid produced liver damage as shown by an increased dye retention of 15–30% (fig. 1). However, when tannic acid was applied to denuded surfaces of rats or rabbits, there was no significant evidence of liver damage. Control animals in which saline was applied to denuded surfaces appeared normal.

Effect on the kidney. The effect of tannic acid on water diuresis in rats was determined by means of the method described by Burn (10). Fifty rats in groups of 5 were fed 5 cc. of warm tap water per 100 grams body weight. The animals were placed in metabolism cages and amount and rate of urine excreted was measured by means of the diuresis recorded of Kniazuk (11). Tannic acid was administered by subcutaneous or intraperitoneal injection or was applied to a denuded area on the backs of the rats. In the latter group of animals the tannic acid was applied 1–2 and 24 hours prior to the diuresis experiment. A similar group of rats was treated with saline and served as controls.

From the results shown in figure 2 it is evident that tannic acid depressed the water diuresis markedly when small doses were given intraperitoneally. However, the subcutaneous or topical application of even much larger doses failed to influence the water diuresis significantly.

SUMMARY

1. Tannic acid is definitely toxic for mice and rats when given by intravenous r subcutaneous injection.

2. When applied to deep subcutaneous tissues following surgical removal f the skin, tannic acid was lethal for mice, but not for rats or rabbits. Tannic acid, or possibly its degradation products, could be detected in the urine of all the foregoing animals by chemical tests.

3. Liver damage was produced in rabbits when tannic acid was administered by subcutaneous injection, but not when the drug was applied to denuded surfaces. The production of liver damage therefore appears to depend on the quantity of tannic acid absorbed.

4. When injected intraperitoneally, tannic acid depresses the water diuresis of rats markedly. This depression could not be produced by the subcutaneous injection of large doses, or by applying the drug to denuded surfaces.

ACKNOWLEDGMENT

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TOXICITY AND EFFICACY OF PENICILLIN

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During the past year considerable interest has been focused on a number of chemotherapeutic agents derived from bacteria, actinomycetes and fungi (1, 2, 3, 4). Among those of particular interest is the substance obtained from certain strains of *Penicillium notatum* called penicillin. This substance, first described by Fleming (5) and later by Chain *et al.* (6), and Abraham *et al.* (7), shows remarkable activity against gram-positive bacteria *in vitro* and *in vivo*. In contrast to the properties of gramicidin (8, 9), penicillin is water-soluble and can protect animals infected by the intraperitoneal injection of gram positive bacteria even when administered intravenously or subcutaneously.

The present communication deals with the acute toxicity and the efficacy of penicillin in certain bacterial, virus and protozoan infections.

MATERIALS AND METHODS. These experiments were performed mainly with penicillin[1] (Merck) equivalent to 60 Florey Units[2] per milligram of solid. In a few experiments, penicillin equivalent to 300 and 400 Florey Units per milligram was used. In each experiment solutions were prepared immediately before use by dissolving the solid in sterile distilled water.

The bacterial *in vitro* and *in vivo* experiments were performed with a variety of cultures representing both the gram negative and gram positive organisms. Most experiments were performed with mouse-virulent cultures grown at 37°C for 6 hours in brain-heart infusion media containing 10% blood. The anaerobic strains were grown under the same conditions using brain heart infusion media supplemented with 0.1% agar-agar.

Both the epidemic influenza virus (PR8) and the *Trypanosoma equiperdum* cultures were maintained by serial passage twice weekly in Swiss mice (Carworth CFW).

TOXICITY EXPERIMENTS. These were performed by injecting penicillin intravenously or subcutaneously into mice in the form of a 10% aqueous solution. Single doses of 0.5, 1.0, 1.5 and 2.0 grams per kgm. were given intravenously, being equal to 30, 60, 90 and 120 thousand Florey units per kgm. animal body weight. The solution was given intravenously at the rate of 0.1 cc. per minute. The subcutaneous toxicity experiments were performed by administering penicillin at 3 hour intervals day and night over a 5 day period. The total daily doses given were 0.8, 1.6 and 3.2 grams per kgm. which were approximately 16, 32 and 64 times the effective penicillin dose respectively. By the use of this procedure the toxicity of penicillin was determined in the same manner as employed in determining its *in vivo* efficacy. In both of the foregoing experiments, observations were made at frequent intervals over a 5 day period and then once daily

[1] The material used in these experiments was prepared by Dr. R. L. Peck from cultures supplied by Dr. J. W. Foster of the Research Laboratory of Merck & Co. Inc.

[2] A Florey unit is that amount of penicillin which when dissolved in 50 cc. of meat extract broth just inhibits completely the growth of the test strain of *Staph. aureus*.

for an additional 10 days. The results of these experiments are presented in tables 1 and 2.

Shortly following the intravenous infection of 0.5 gram (30,000 units) per kgm. or more, mice became inactive and appeared quite depressed. This was followed by a period of increased activity characterized by constant movement with some evidence of respiratory embarrassment. All mice had watery eyes and with the larger dose levels the veins of both the ears and the cornea appeared dilated. Tissues of all mice were yellow, due probably to a diffusion of penicillin. With the higher dose levels the body temperature dropped significantly within 30 minutes following the intravenous injection. When daily doses of 3.2 grams

TABLE 1

Acute intravenous toxicity of penicillin in mice

DOSE	DOSE	NO. OF MICE	NO DEAD AFTER (DAYS)										PER CENT DEAD
			1	2	3	4	5	6	7	8	9	10	
gm/kgm/day	**units/kgm/day*												
0.5	30,000	10	0	0	0	0	0	0	0	0	0	0	0
1.0	60,000	10	4	1	0	1	0	0	0	0	0	0	60
1 5	90,000	10	9	0	0	0	0	0	0	0	0	0	90
2.0	120,000	10	10	0	0	0	0	0	0	0	0	0	100

* Florey units.

TABLE 2

Subcutaneous toxicity of penicillin in mice

*DOSE	DOSE	NO. DEAD AFTER (DAYS)										TOTAL NO. DEAD
		1	2	3	4	5	6	7	8	9	10	
gm/kgm./day	*†units/kgm./day*											
0.8	48,000	0	0	0	0	0	0	0	0	0	0	0
1 6	96,000	0	0	0	0	0	0	0	0	0	0	0
3.2	192,000	0	2	0	0	2	0	0	0	0	0	4

* Dosed for 5 days. 10 mice per dose level.

† Florey units.

(192,000 units) per kilogram were given subcutaneously in divided doses at 3 hour intervals, over the 5 day period, all the mice appeared sick during the period of treatment and 4 out of 10 mice died. All the latter mice showed signs of necrosis at the site of injection indicating that penicillin in high concentrations is irritating. Mice treated in the same manner with daily doses of 0.8 or 1.6 grams per kgm. appeared normal with the exception of a slight local reaction at the site of the injection. With the exception of this local effect, gross examination of the tissues of mice sacrificed after the observation period did not reveal any significant findings. All tissues were retained for histological study[3].

[3] These will be reported elsewhere by Dr. Henry Siegel.

Acute intravenous toxicity experiments were also performed in a small series of mice using a more purified preparation equal to 400 Florey Units per milligram. Single doses of 0.5, 1.0 and 1.5 gram per kgm. produced approximately the same toxic signs observed with the crude preparation although none of the mice died. Doses above this level were lethal. It seems therefore that on the basis of weight, a purified penicillin preparation is somewhat less toxic than crude penicillin in spite of the marked increase in antibacterial activity. It is not unlikely, therefore, that the difference between the toxic and the effective dose of pure penicillin may be even greater than that reported in this communication.

EFFICACY EXPERIMENTS. The *in vitro* effect of penicillin in bacterial infection was studied using the method described by Lockwood (10) which resembles as closely as possible conditions *in vivo*. The method consists essentially in placing bacteria and penicillin in small test tubes which are sealed and rotated slowly on a mixing machine at 37°C. At a given time interval the tubes are opened and the number of viable bacteria determined by making pour plates of 0.1 cc. of the contents. In all experiments, 2.0 cc. of defribinated blood were used as the test medium. Under these conditions, penicillin in dilutions of 1 to 2,000,000 was rapidly bactericidal to a strain of *Strep. hemolyticus*. *Staph. aureus* and *Diplo. pneumoniae* were somewhat more resistant (table 3). In dilutions of 1 to 5,000,000 penicillin appeared to be primarily bacteriostatic to the foregoing bacterial strains. A strain of *Strep. viridans* was much more resistant to penicillin requiring one part in 50,000 to produce a killing effect.

In a second series of experiments penicillin was incorporated into melted blood agar which was permitted to solidify. Next, the surface of the agar plate was streaked with a variety of gram positive and gram negative pathogenic bacteria and incubated at 37°C. for 24 hours. The plates were then examined for the degree of growth as compared with control blood agar plates without penicillin.

Results of these experiments indicated that the contrast between the action of penicillin on gram negative and gram positive bacteria is striking (table 4). Dilutions of 1:1000 were required to inhibit the growth of most gram-negative strains whereas 1:1,000,000 inhibited all the gram positive strains with the exception of *Strep. viridans*, *Strep. lactis* and one strain of *Staph. aureus*. It is of interest to note that even among the gram-negative or the gram-positive strains there occur considerable differences in resistance to penicillin. Thus two strains of *Streptococcus hemolyticus* (1685, C-203) were completely inhibited at 1:2,000,000 whereas *Streptococcus viridans* or *Streptococcus lactis* were only partially inhibited at 1:128,000. Likewise, of the gram negative group, strains of *Escherichia coli* and *Bacterium aerogenes* were unaffected by penicillin, whereas strains of the *Salmonella* and bacillary dysentery group were inhibited in dilutions of 1:1000 to 1:4000.

Finally, the bacteriostatic effect of penicillin against anaerobic bacteria was determined by means of a slight modification of Kolmer's method (11). Details of this procedure have been described in a recent report (12). Briefly, it consists in determining the smallest quantity of penicillin required to inhibit the

growth of anaerobic bacteria in brain-heart infusion supplemented with 0.1% agar as the test medium. The results of these experiments are shown in table 5.

TABLE 3

Efficacy of penicillin in blood in vitro (rotating rock technique)

TUBE NO.	MICROGRAMS OF PENICILLIN PER CC OF BLOOD	NUMBER OF VIABLE BACTERIA PER 0 001 CC. OF BLOOD					
		Time in hours					
		0	1	3	6	10	24
Streptococcus hemolyticus (C-203)							
1	0.1	280	748	1920	∞	∞	∞
2	0.2	240	472	2700	∞	∞	∞
3	0.3	180	512	2480	∞	∞	∞
4	0.4	210	196	62	41	0	0
5	0.5	220	89	10	0	0	0
6	0.6	205	27	0	0	0	0
7	0.7	240	4	0	0	0	0
8	0.8	210	0	0	0	0	0
9	0	452	∞	∞	∞	∞	∞
10	0	680	∞	∞	∞	∞	∞
Staphylococcus aureus (Smith)							
1	0.5	460	100	780	3840	∞	∞
2	0.7	440	130	280	590	2720	∞
3	0 9	400	160	125	75	10	0
4	1.1	390	130	0	0	0	0
5	1 3	420	160	0	0	0	0
6	0	430	310	∞	∞	∞	∞
7	0	410	270	∞	∞	∞	∞
Pneumococcus Type I							
1	0 7	320	720	2400	∞	∞	∞
2	0 9	300	320	780	∞	∞	∞
3	1.1	280	200	74	0	0	0
4	1.3	380	280	72	0	0	0
5	0	320	750	∞	∞	∞	∞
6	0	290	760	∞	∞	∞	∞
Streptococcus viridans							
1	2.5	280	720	2400	∞	∞	∞
2	5.0	290	430	1020	∞	∞	∞
3	10 0	320	380	2400	∞	∞	∞
4	20 0	290	200	20	0	0	0
5	25 0	310	360	16	0	0	0
6	0	380	794	∞	∞	∞	∞
7	0	296	820	∞	∞	∞	∞

It is apparent that penicillin is very effective against *Cl. welchii*, *Cl. tetani*, *Cl. botulinum* and *Cl. chauvoii*.

Similar to the findings of Chain, definite morphological changes in cultures treated with penicillin were observed. These changes also occurred in concentrations where penicillin had no apparent bacteriostatic effect.

TABLE 4

In vitro activity of penicillin (Agar plate method)

DILUTION OF PENICILLIN	*Strep. hemolyticus 1685*	*Strep. hemolyticus C-203*	*Strep. viridans*	*Strep. lactis*	*Staph. aureus (S.D.)*	*Staph. aureus (F.D.A.)*	*Staph. aureus (H.S.)*	*Staph. aureus (S.)*	*Staph. aureus (Smith)*	*Diplo. pneumoniae type I*	*S. aertrycke*	*S. enteriditis*	*S. schottmülleri*	*B. flexneri*	*B. shiga*	*B. sonne*	*B. flexneri "D"*	*P. lepiseptica*	*E. Coli*	*A. aerogenes*
1:1000											0	0	0	0	0	0	0	0	3	4
1:2000											0	0	0	1	0	2	1	0	4	4
1:4000											1	0	0	3	2	4	3	1	4	4
1:8000											2	2	2	3	3	4	3	3	4	4
1:16,000											4	3+	3	3	4	3+	3	3+	4	4
1:32,000											4	4	4	3	4	4	4	3+	4	4
1:64,000			0	0							4	4	4	4	4	4	4	3+	4	4
1:128,000			1	1							4	4	4	4	4	4	4	4	4	4
1:256,000			2	2							4	4	4	4	4	4	4	4	4	4
1:512,000			3	3							4	4	4	4	4	4	4	4	4	4
1:1,000,000	0	0	3	3	0	0	3	0	0	0										
1:2,000,000	0	0	4	4	1	1	4	0	0	0										
1:4,000,000	0	0	4	4	2	0	4	0	0	0										
1:8,000,000	0	0	4	4	3+	2	4	3	3	1										
1:16,000,000	3	3	4	4	3	3	4	4	3	2										
1:32,000,000	3+	4	4	4	4	4	4	4	4	4										
Control	4	4	4	4	4	4	4	4	4	4										

Key: Growth: 0 = negative; 1 = very poor; 2 = fair; 3 = good; 4 = excellent growth.

TABLE 5

Bacteriostatic efficacy of penicillin against anaerobic bacteria

DILUTION OF PENICILLIN	*Cl. welchii*	*Cl. chauvoii*	*Cl. tetani*	*Cl. botulinum*
1:50,000	−	−	−	−
1:60,000	−	−	−	−
1:70,000	−	−	−	−
1:80,000	−	−	−	−
1:90,000	−	−	−	+
1:100,000	−	−	−	+
1:200,000	±	+	−	+
1:400,000	±	+	±	+
Control	+	+	+	+

Key: + = growth; − = no growth.

In Vivo EXPERIMENTS. The effect of penicillin in gram-positive and gram-negative bacterial infections was studied in mice. Mouse-virulent strains of *Streptococcus hemolyticus* 1685, *Diplococcus pneumoniae* (Type I), *Staphylococcus*

aureus (Smith), *Salmonella artrycke*, *Salmonella schottmülleri* and *Bacterium shiga* were used. The animals were infected by the intraperitoneal injections of 0.5 cc. of a 10^{-4} dilution of a six hour culture of the above organisms. With all strains, this quantity of culture was equal to approximately 10,000 to 100,000 lethal doses, as determined by titration in mice. In the staphylococcal, *S. artrycke* and *S. schottmülleri* infections, it was found necessary to employ 4%

TABLE 6

Efficacy of penicillin in streptococcal infections in mice

Organism: *Streptococcus hemolyticus* #1685
Age of culture: 6 hours
Infection: 0.5 cc. of a 10^{-4} culture dilution in broth
Treatment: Penicillin given subcutaneously and sulfanilamide given orally immediately after the inoculation of bacteria
Interval between treatments: Every 3 hours day and night for 5 days

DRUG	DOSE	CULTURE DILUTION	DOSE	NUMBER SURVIVING IN DAYS									
				1	2	3	4	5	6	7	8	9	10
	mgm / day		**units/ day*										
Penicillin	0.0625	10^{-4}	3.75	0									
	0.125	10^{-4}	7.5	16	0								
	0.250	10^{-4}	15	18	4	1	1	1	1	1	1	0	0
	0.500	10^{-4}	30	20	4	0							
	1.0	10^{-4}	60	20	20	18	15	15	15	15	13	9	9
	2.0	10^{-4}	120	20	20	20	20	20	20	20	20	20	20
	4.0	10^{-4}	240	20	20	20	20	20	20	20	20	20	20
Sulfanilamide	0.5	10^{-4}		20	0	0	0	0	0	0	0	0	0
	1.0	10^{-4}		20	20	20	10	8	8	5	4	1	1
	2.0	10^{-4}		20	20	20	20	20	20	20	20	14	3
	4.0	10^{-4}		20	20	20	20	20	20	20	19	19	16
	8.0	10^{-4}		20	20	20	20	20	20	20	20	20	18
	16.0	10^{-4}		20	20	20	20	20	20	20	20	20	20
Controls	0	10^{-4}		0									
	0	10^{-5}		0									
	0	10^{-6}		0									
	0	10^{-7}		0									
	0	10^{-8}		0									

* Florey units.

gastric mucin whereas the streptococci and pneumococci were injected suspended in broth. Treatment with penicillin was given subcutaneously, immediately after the inoculation of bacteria and then repeated at 3 or 6 hour intervals day and night over a 5 day period. Observations were made for an additional 10 day period. For comparison, similar experiments were performed with sulfanilamide, sulfathiazole and sulfadiazine in which infected mice were treated orally at 3 hour intervals. The results of these experiments are given in tables 6, 7,

and 8. For convenience only the result at daily intervals is presented in these tables.

It is apparent that penicillin in daily doses of 2–4 mgm. (120–240 units) per 20 gram mouse afforded excellent protection to mice infected with the gram-positive organisms when treated at 3 or 6 hour intervals. Smaller doses of 0.25–0.5 mgm. (15–30 units) showed some protection but eventually the mice died.

TABLE 7

Efficacy of penicillin in pneumococcal infections in mice

Organism: *Pneumococcus Type I #37*
Age of culture: 6 hours
Infection: 0.5 cc. of a 10^{-4} culture dilution in broth
Treatment: Penicillin given subcutaneously and sulfadiazine given orally immediately after the inoculation of bacteria
Interval between treatments: Every 3 hours day and night for 5 days

DRUG	DOSE	CULTURE DILUTION	DOSE	NUMBER SURVIVING IN DAYS									
				1	2	3	4	5	6	7	8	9	10
	mgm/day		**units/day*										
Penicillin	0.0625	10^{-4}	3.75	0									
	0.125	10^{-4}	7.5	0									
	0.250	10^{-4}	15	0									
	0.500	10^{-4}	30	0									
	1.000	10^{-4}	60	13	5	4	4	4	4	4	4	4	4
	2.000	10^{-4}	120	20	20	20	20	20	20	20	20	20	20
	4.000	10^{-4}	240	20	20	20	20	20	20	20	20	20	20
Sulfadiazine	0.5	10^{-4}		0									
	1.0	10^{-4}		0									
	2.0	10^{-4}		12	0								
	4.0	10^{-4}		14	12	0							
	8.0	10^{-4}		20	20	20	15	9	7	0			
	16.0	10^{-4}		20	20	20	20	20	12	8	8	8	3
Controls	0	10^{-4}		0									
	0	10^{-5}		0									
	0	10^{-6}		1	0								
	0	10^{-7}		3	0								
	0	10^{-8}		0									

* Florey units.

In general the protection afforded by penicillin was greater than that of the sulfonamides, particularly if the observation period was extended over a 10 day period. Furthermore, the protection afforded by penicillin appeared to be more permanent than that of the sulfonamides suggesting that penicillin is more bactericidal *in vivo*. An additional advantage of penicillin appears to be in the rapidity with which it is absorbed. Mice infected with 10,000 L.D. of pneumococci and treated 6 to 7 hours later survive the infection. Under similar conditions the

sulfonamides are not effective. Penicillin was not effective against the gram-negative bacterial infections.

Tuberculous Infections in Mice. The effect of penicillin against tuberculosis was studied in mice using two experimental procedures. The first group of experiments consisted in suspending *Mycobacterium tuberculosis* (avian) in 1.6% aqueous solution of penicillin for 48 hours at 37°C. The penicillin-bacterial

TABLE 8

Efficacy of penicillin in staphylococcal infections in mice

Organism: *Staphylococcus aureus* (Smith)
Age of culture: 6 hours
Infection: 0.5 cc. of a 10^{-4} culture dilution in 4% mucin
Treatment: Penicillin given subcutaneously and sulfathiazole given orally immediately after the inoculation of bacteria
Interval between treatments: Every 3 hours day and night for 5 days

DRUG	DOSE	CULTURE DILUTION	DOSE	NUMBER SURVIVING IN DAYS									
				1	2	3	4	5	6	7	8	9	10
	mgm./day		**units/day*										
Penicillin	0.0625	10^{-4}	3.75	0									
	0.125	10^{-4}	7.5	4	0								
	0.250	10^{-4}	15	20	12	1	1	1	1	1	1	1	1
	0.500	10^{-4}	30	20	12	0							
	1.000	10^{-4}	60	20	20	20	20	20	20	20	20	20	20
	2.000	10^{-4}	120	20	20	20	20	20	20	20	20	20	20
	4.000	10^{-4}	240	20	20	20	20	20	20	20	20	20	20
Sulfathiazole	0.5	10^{-4}		17	9	4	3	3	0				
	1.0	10^{-4}		20	18	18	18	18	18	18	18	18	14
	2.0	10^{-4}		20	20	20	20	20	18	16	16	12	12
	4.0	10^{-4}		20	20	20	20	20	20	20	20	20	20
	8.0	10^{-4}		20	20	20	20	20	20	20	20	20	20
	16.0	10^{-4}		20	20	20	20	20	20	20	20	20	20
Controls	0	10^{-4}		0									
	0	10^{-5}		0									
	0	10^{-6}		0									
	0	10^{-7}		0									
	0	10^{-8}		0									

* Florey units.

mixture was then injected intravenously into mice in doses of 0.01, 0.1 and 1.0 mgs. of tubercle bacilli per 20 gram body weight and the animal was killed after 3–4 weeks and examined for infection. Under these conditions the tubercle bacilli were exposed to a maintained concentration of penicillin much greater than that obtained in the course of experimental *in vivo* tests.

In the second series of tests the organisms were suspended in physiological saline and injected intravenously in doses of 0.001, 0.1 and 1.0 mgm. per 20 gram

animal. The infected mice were then treated with penicillin, at 3 hour intervals for 3 weeks with a total dose of 1000 units per mouse per day.

In both experiments penicillin had no apparent effect on the course of the infection in mice. Mice killed after a period of 3 weeks presented the same gross pathological picture as the untreated control groups.

Use of Penicillin in Virus Infections. The virus PR8 of epidemic influenza was used in this series of experiments. Infected mouse lungs obtained from the 487 mouse passage were ground in a porcelain mortar with a small quantity of Alundum sand. A 20% suspension by weight of ground mouse lungs was prepared in 10% horse serum and the mixture centrifuged for 10 minutes at 1000 R.P.M. The supernatant fluid was diluted serially in 10 fold dilutions to 10^{-5}. The mice were inoculated intranasally while under light ether anesthesia.

The first groups of 50 mice were inoculated with 0.05 cc. of the 10^{-5} dilution and treated subcutaneously with penicillin at 3 hour intervals day and night until death. Each mouse received 1000 units of penicillin daily during the course of the test. In the second group of mice the penicillin and the supernate containing the virus were mixed *in vitro* at 37°C for 1 hour before intranasal inoculation in mice. The third group served as untreated controls.

All three groups of mice died within 7 to 8 days indicating that penicillin had essentially no effect in this infection. The macroscopic lung lesions of all groups were also of the same order.

Trypanosomiasis. Mice were infected intraperitoneally with a killing dose of *Trypanosoma equiperdum* and treated subcutaneously at 3 hour intervals with penicillin. As in the virus infections, the mice were treated with 1000 units of penicillin daily until the death of all the animals. A second group of 50 mice were treated with 10 mgs. of tryparsamide daily while a third group remained untreated and served as controls.

Here again penicillin had no effect on the course of the experimental infection. All three groups of mice died at the same rate, and examination of blood smears during the course of treatment indicated that the infection progressed equally in both the control and penicillin treated mice. Tryparsamide protected all the mice under these experimental conditions.

COMMENTS. Penicillin is apparently a highly effective chemotherapeutic agent in infections caused by gram-positive bacteria. The range between the lethal and effective dose is considerable, particularly if the toxicity is determined under the same conditions as employed in determining therapeutic efficacy. The experiments presented suggest that penicillin is superior to sulfanilamide and its derivatives in streptococcal, pneumococcal and staphylococcal infections in mice.

Penicillin did not appear to have any effect on infections caused by *Mycobacterium tuberculosis* (avian), *Trypanosoma equiperdum* or the influenza virus PR8.

It should be pointed out that the penicillin available for experimental and clinical study at present is relatively impure.

Additional experiments will be undertaken as soon as the pure penicillin is available.

SUMMARY

1. Crude penicillin is toxic for mice when given intravenously in single doses of 0.5, 1.0, 1.5 and 2.0 grams per kgm. More highly purified preparations appeared to be less toxic than the crude preparation when compared on a weight basis.

2. When given subcutaneously penicillin is well tolerated by mice in daily doses of 1.6 grams per kgm. over a 5 day period. Under the same conditions 3.2 grams per kgm. are lethal for some mice.

3. The toxic dose of crude penicillin appears to be about 64 times the effective dose as determined by subcutaneous injection in mice.

4. On the basis of weight, penicillin appears to be more effective than sulfanilamide and its derivatives in streptococcal, pneumococcal and staphylococcal infections in mice.

5. Penicillin had no apparent effect in experimental infections caused by *Mycobacterium tuberculosis*, *Trypanosoma equiperdum* or the influenza virus PR8.

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THE EFFECT OF DRUGS ON THE PULMONARY AND SYSTEMIC ARTERIAL PRESSURES IN THE TRAINED UNANESTHETIZED DOG

RENIN, ANGIOTONIN, ADRENALIN, PITRESSIN, PAREDRINE, DIGITALIS, ACETYLCHOLINE, PAPAVERINE, HISTAMINE, AMYL NITRITE AND AMINOPHYLLIN*

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General considerations. Considerable knowledge has been accumulated concerning the action of various drugs on the preliminary circulation based, for the most part, on studies made in anesthetized animals with the chest opened. The subject has been adequately reviewed by Wiggers (1), Daly (2) and Hochrein (3) among others. However, little experimental work has been done using the intact animal, particularly in the absence of anesthesia. While the knowledge obtained in the anesthetized animal with chest open has helped to reveal the mode of action of many of these drugs, it has given no direct information on the net effects in the unanesthetized animal. The development of a cannula technique (4) permitting simultaneous analyses of blood pressure in the pulmonary and systemic circuits, has made it possible to measure the effects of a number of drugs upon the pulmonary and systemic arterial pressures in unanesthetized trained dogs. When integrated with knowledge previously obtained by others on the anesthetized animal, a more adequate understanding of drug action becomes available. This information is thus more directly applicable to problems of drug therapy in man.

In themselves these experiments do no more than reveal the integration of the various factors controlling the pulmonary blood pressure under the influence of the drug employed. Other information must be analyzed to evaluate the action of the drug on the individual factors contributing to the total effect. Some information can be ascertained from previous work, other aspects require further investigation. The consideration of the action of each of the drugs employed in this study will be facilitated by a brief resumé of the several factors which influence pulmonary arterial pressure. These fall into four main groups, viz., (*1*) the mechanical action of respiration, (*2*) the effect of the relative contractile power of the right and left ventricles, (*3*) the passive effect of large changes in circulating blood volume, and (*4*) the effect of direct change in the caliber of the pulmonary vessels by virtue of their *vasodilatation or vasoconstriction*.

Respiration may mechanically alter pulmonary arterial blood pressure in at least one of two ways. It may act by virtue of changes in intrapulmonary and intrapleural pressure

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which are directly reflected in the pulmonary arterial pressure or indirectly by varying the pulmonary vascular resistance as a result of different degrees of compression on the smaller vessels and pulmonary veins. It may also act through the traction effect on the intrapulmonary vessels which decreases the total pulmonary peripheral resistance at certain lung sizes and increases it at others by altering the net product of length and cross section area of the smaller pulmonary vessels. It is doubtful whether in the intact normal animal this latter factor plays any significant rôle.

A much more important factor influencing pulmonary arterial pressure is the relative contractile power of the two ventricles. Normally the two are so adjusted that the amount of blood in the pulmonary vascular bed remains fairly constant except for transient periods following changing venous return to the right heart, and even here the changes in pulmonary pressure are not large (5). However, when the contractile power of the two ventricles is altered, as for example, by the administration of certain drugs, it is possible that the enhancement or depression may be unequal in the two ventricles This would lead to depletion or, more often, congestion in the pulmonary vascular bed, with a resultant change in pulmonary vascular pressure. Such changes in the pulmonary arterial pressure are therefore passive phenomena due to changes in the blood content of the lungs.

Similar passive changes in pulmonary arterial pressure would be expected to occur whenever sizeable changes in the circulating blood volume occur, e g , in shock, hemmorhage or plethora. In some instances drug action might also alter pulmonary aterial pressure primarily by an action on the capacity of the systemic blood reservoirs and its peripheral vascular bed secondarily affecting the blood content of the lungs.

Before the action of a drug can be attributed to a direct effect on the vascular tone of the pulmonary vessels, passive actions must be excluded. Even after elimination of these passive factors, other secondary actions must be excluded before a direct effect on the vascular tone of the pulmonary vessels is indicated. For example, by an action on respiration, a drug may act to alter the blood content of CO_2 and O_2 and these changes in the blood may in turn result secondarily in a direct or reflexogenic pulmonary vasomotor change. Likewise, the drug might cause a marked decrease in cardiac output with resultant ischemia, or it might set off humoral mechanisms from other organs which might lead to a secondary direct or reflexogenic pulmonary vasomotor response. Reflexogenic responses on the part of the pulmonary vasculature might also result secondarily from actions of the drug altering venous return or systemic arterial pressure. Only after these secondary effects are definitely excluded is it justifiable to ascribe the vasomotor change found to a direct effect of the drug on the blood vessels, on its efferent innervation, on the vasomotor center or on the receptors giving rise to the reflexogenic response.

Despite the fact that it is not always possible to account for the cause of the pressure changes, knowledge of the pressure changes is in itself of value when obtained on trained unanesthetized animals. In the present report, we propose to deal separately with each drug studied, first reporting our findings and then attempting to account for its action insofar as known facts permit.

METHODS. Large dogs (weighing around 20 kilos) were used in these experiments. A modified London cannula (4) was attached to the pulmonary artery so that a needle could later be inserted into the pulmonary artery to record its pressure without opening the chest. The procedure followed in this operation and in the post-operative care was that developed in this laboratory (4). The flange of the cannula is placed beneath the skin and serves as a guide for the specially constructed long (12 cm) manometer needle After recovery from the operation, the animal is trained to lie quietly while simultaneous pulmonary and systemic arterial pressures are recorded All the dogs used in this study were trained so that they were not excited by these manipulations. Blood pressures were recorded with Hamilton needle manometers (6) as previously described by us (7), the systemic pressure

being recorded from the femoral artery. In normal trained dogs the systemic pressure has been found to equal about 150/80 mm. Hg (7) and the pulmonic about 30/10 mm. Hg (4). Blood pressure readings were taken before, during and following drug injection and usually continued until the pressures had returned to, or almost to, control levels. Only those records were used in which the evidence from pulse contour suggested that the needle was not clotted and that it was free in the arterial lumen. In some records secondary oscillations developed as a result of vibrations set up in the needle by impact upon the cannula as the heart moved during its cycle. When such irregularities prevented the measurement of pressure levels, the record was discarded. The systolic, diastolic and pulse pressures of the pulmonic and systemic arteries were measured after calibration. Heart rate changes were determined from the records as were effects on the normal pulse arrhythmia (sinus arrhythmia). Observations were also made on the action of the drugs on respiration and on the general reaction of the animal. In so far as possible the dosage of the drugs was made roughly comparable to clinical dosage on the basis of body weight, although for special reasons larger or smaller doses were sometimes used.

RESULTS. *Renin and angiotonin.* It has been thoroughly established that both renin and angiotonin are powerful vasoconstrictors of the systemic vessels (8) and they have been implicated in the production of nephrogenic hypertension. We have found that when systemic hypertension is produced by partial renal artery occlusion, the hypertension is confined to the systemic circuit and is not shared by the pulmonic (4). In the present study, it was our intent to determine whether pulmonary hypertension occurred concomitant with the acute systemic hypertension produced by intravenous injection of renin and angiotonin. Seven experiments on 3 dogs were carried out with renin[1] and five experiments on two dogs with angiotonin[1]; several other experiments with renin were discarded because the quantity used was insufficient to give a systemic pressure elevation. The renin was injected intravenously in 1 to 2.5 cc. quantities over a period of 5 sec. or less, the amount varying with the potency of the product. Angiotonin was injected intravenously in 0.1 to 0.2 cc. quantities over a like period of time. Typical experiments are illustrated in figs. 1 and 2.

No demonstrable significant change was observed in the animal's behavior or in its respiration. In all but one of the renin experiments and all but one of the angiotonin ones, the heart rate slowed. This slowing of heart rate is in accord with the observation on angiotonin in man (9, 10) and appears to be a reflex from the carotid sinus resulting from the elevation in systemic pressure. It is most marked at the peak of the pressure rise and is associated with accentuation of the respiratory sinus arrhythmia also suggesting increased vagal tone.

In the case of renin, the rise in systemic diastolic blood pressure averaged 47 mm. Hg and the systolic rise was even greater since the pulse pressure increased in 3 instances. In the case of angiotonin, the systemic diastolic pressure rise averaged 39 mm. Hg and the systolic rise was even greater since the pulse pressure increased in 4 cases. The rise in systemic pressure was more rapid in the

[1] We are grateful to Dr. I. H. Page of Indianapolis for generous supplies of angiotonin and renin, and to Dr. I. A. Mirsky, Cincinnati and Dr. L. Leiter of Chicago for other batches of renin.

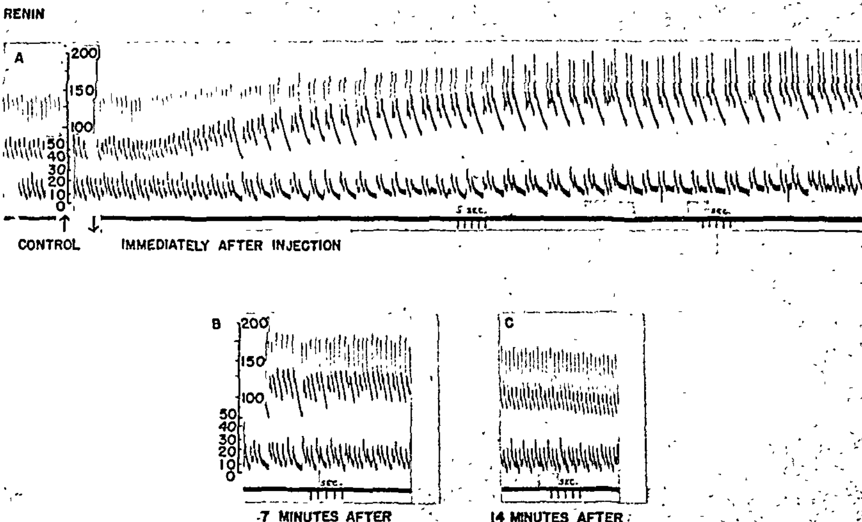

FIG. 1. TYPICAL RECORD OF EFFECT OF RENIN 1 cc. (PAGE) INJECTED INTRAVENOUSLY

A is the control taken before, effect during (indicated by two arrows) and immediately after injection. *B* and *C* were taken respectively 7 and 14 minutes after injection. Upper curve is femoral arterial pressure; lower curve, pulmonary arterial pressure. Base line is below. Time is indicated in seconds. The calibration curves for each record in mm. Hg. are shown on the left. *Discussed in text.*

being recorded from the femoral artery. In normal trained dogs the systemic pressure has been found to equal about 150/80 mm. Hg (7) and the pulmonic about 30/10 mm. Hg (4). Blood pressure readings were taken before, during and following drug injection and usually continued until the pressures had returned to, or almost to, control levels. Only those records were used in which the evidence from pulse contour suggested that the needle was not clotted and that it was free in the arterial lumen. In some records secondary oscillations developed as a result of vibrations set up in the needle by impact upon the cannula as the heart moved during its cycle. When such irregularities prevented the measurement of pressure levels, the record was discarded. The systolic, diastolic and pulse pressures of the pulmonic and systemic arteries were measured after calibration. Heart rate changes were determined from the records as were effects on the normal pulse arrhythmia (sinus arrhythmia). Observations were also made on the action of the drugs on respiration and on the general reaction of the animal. In so far as possible the dosage of the drugs was made roughly comparable to clinical dosage on the basis of body weight, although for special reasons larger or smaller doses were sometimes used.

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case of angiotonin than with renin, the peak was reached sooner and the effect was dissipated earlier. These effects with angiotonin are briefer, though similar to the effects in man (11). The rise in pulse pressure may be in part contributed to by the slowing of the heart, but the correlation is too imperfect for this to be the sole factor. A change in the elastic reservoir capacity of the arterial tree resulting from the rise in pressure probably contributes to this increase in pulse pressure. The literature on cardiac output, which might affect pulse pressure, is conflicting. In man observations have shown that minute cardiac output after angiotonin is unchanged or actually decreased (9, 10). In the intact dog, renin does not alter the cardiac output (12), in the cat the output of the isolated heart is also unchanged or increased after renin (13), but in the cat heart-lung both renin and angiotonin usually, but not always, increase the output (13); on occasion angiotonin decreases the output in this preparation (13). Similar variable results occur with renin and angiotonin on coronary vascular tone (14).

A proportional rise in pulmonary arterial pressure was observed accompanying the rise in systemic pressure with both renin and angiotonin. Thus the rise in diastolic pressure of the pulmonary artery averaged 9 mm. Hg for renin, and 12 mm. Hg for angiotonin. However, this represents a greater percentage increase in pulmonary pressure than in the systemic since the control diastolic value in the latter is 80 mm. while in the former it is about 10 mm. With angiotonin the pressure rise in the pulmonary artery occurred almost simultaneously with the systemic rise and the pressure in both fell at the same time. In the case of renin, the pulmonary pressure rise lagged behind the systemic, a lag which varied with the rate of systemic pressure elevation. Further, with renin, the pressor effect in the pulmonary circuit disappeared sooner than in the systemic.

The pulse pressure in the pulmonary artery rose roughly parallel with that of the systemic artery. However, here again, there were greater elevations in pulse pressure in the pulmonary than in the systemic artery when considered in terms of the percentage increase in each circuit. The same factors, heart slowing and an increase in the elastic reservoir capacity as the pressure level rises, apparently operate to increase the pulse pressure in the pulmonary as in the systemic vessels.

The rise in pulmonary diastolic pressure might be ascribed to two factors which do not involve pulmonary vasoconstriction. The first is the increase in venous return to the heart brought about by the shrinkage of the peripheral vascular bed causing a redistribution of blood between the systemic and the pulmonary circuit and leading to pulmonary congestion and hypertension. The second is a decrease in power of the heart. For the latter, some evidence is available as far as angiotonin is concerned in man (10). If this affects the left ventricle more than the right then pulmonary congestion and hypertension will result. Either of these two mechanisms will explain the pulmonary arterial pressure changes in our experiments as well as the decreased vital capacity and rise in systemic venous pressure reported after angiotonin in man (10).

In view of these indirect actions, more direct proof must therefore be found

than is now available if a pulmonary vasoconstrictor action by renin and angiotonin is to be proved. The absence of any rise in pulmonary pressure in the more chronically developing nephrogenic hypertension (4) would indicate either that renin and angiotonin are not involved in renal hypertension, or, if involved, that compensatory mechanisms operating over a longer period of time serve to prevent any permanent changes in pulmonary pressure.

Adrenalin. An elevation of pulmonary arterial pressure after adrenalin has been noted in the anesthetized animal (1, 15, 16, 17). Our results on 4 experiments using three trained unanesthetized dogs are in accord with these previous observations. In these experiments 2 cc. of 1:10,000 adrenalin[2] was administered intravenously over a period of 5 sec. A typical experiment is shown in figure 3. It was found that the pressor effects on the systemic circuit are similar to those reported in the literature for animals (19) and for man (20, 21, 22). There was also an indication of respiratory stimulation in our records. Slowing of heart rate such as we observed is well established in animals (19) and after intravenous injection in man (23).

The pulmonary arterial pressure elevations in our experiments were proportional to the systemic but much smaller in extent, and the rise in the former circuit lagged slightly, and returned sooner to normal. The increase in pulse pressure was parallel in the two circuits and seemed primarily attributable to the slowing of the heart in our experiments, although such an increase has been observed in the systemic circuit even when cardiac slowing was absent. The pulse pressure rise may also be attributed in part to the increase in cardiac stroke volume found to occur in the animal (24) and in man (21, 25). It may also, in part, be due to the characteristics of the elasticity curve of the two arterial systems by which a higher mean pressure, even with the same stroke volume, leads to an augmented pulse pressure.

There is no need to invoke pulmonary vasoconstriction to explain this effect of adrenalin, a subject which is at present highly controversial (cf. Daly (2)). In fact, the observation of Johnson *et al.* (17) and Hamilton, Woodbury and Vogt (18) that the pulmonary *venous* pressure shares in this rise tends to disprove the importance of pulmonary vasoconstriction. It does not, however, follow that this pulmonary congestion, evidenced in man by a decreased vital capacity (26), is due to left heart failure since most workers have found an increased cardiac output (27). Occasionally, no doubt, left heart failure does occur, but it is more likely that the pulmonary congestion with adrenalin is due to redistribution of blood between the two major circuits. As the action of adrenalin decreases the capacity of the systemic vascular tree, the blood is redistributed to the lungs and blood tends to accumulate there. Such a redistribution of blood will explain all the findings noted on the intact animal including the pulmonary arterial pressure rise without involving the presence of failure of the left heart. While coronary vasodilatation occurs (28) and contributes to the rise in pulmonary arterial pressure in the heart-lung preparation, its effect in the intact animal probably is minimal since any enhancement in the amount of the cardiac

[2] Generously supplied by Parke, Davis and Company.

output via the coronaries will be accompanied by a proportionate decline in the flow via other systemic pathways.

Pitressin In an extensive study on the anesthetized rabbit, cat and dog Holtz (29) found that pitressin (and pituitrin) had different actions in different

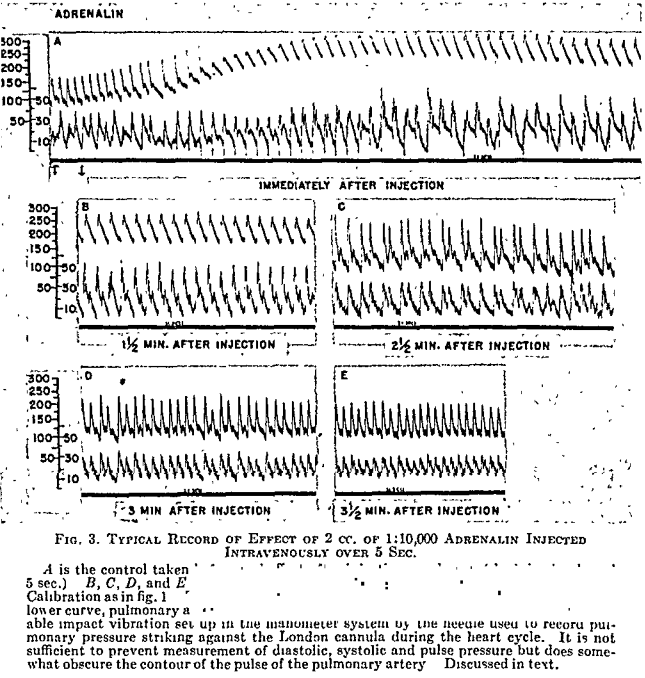

FIG. 3. TYPICAL RECORD OF EFFECT OF 2 CC. OF 1:10,000 ADRENALIN INJECTED INTRAVENOUSLY OVER 5 SEC.

A is the control taken
5 sec.) *B*, *C*, *D*, and *E*
Calibration as in fig. 1
lower curve, pulmonary a
able impact vibration set up in the manometer system by the needle used to record pulmonary pressure striking against the London cannula during the heart cycle. It is not sufficient to prevent measurement of diastolic, systolic and pulse pressure but does somewhat obscure the contour of the pulse of the pulmonary artery Discussed in text.

species. In the cat, these drugs caused a rise in pulmonary arterial pressure, while in the rabbit and dog a fall in pressure occurred. (In the dog, 1 to 3 pressor units of pitressin were employed)

Our results with pitressin in the unanesthetized trained dog differ from Holtz's. We have tested this drug 7 times in 4 dogs injecting 8 to 20 pressor units of

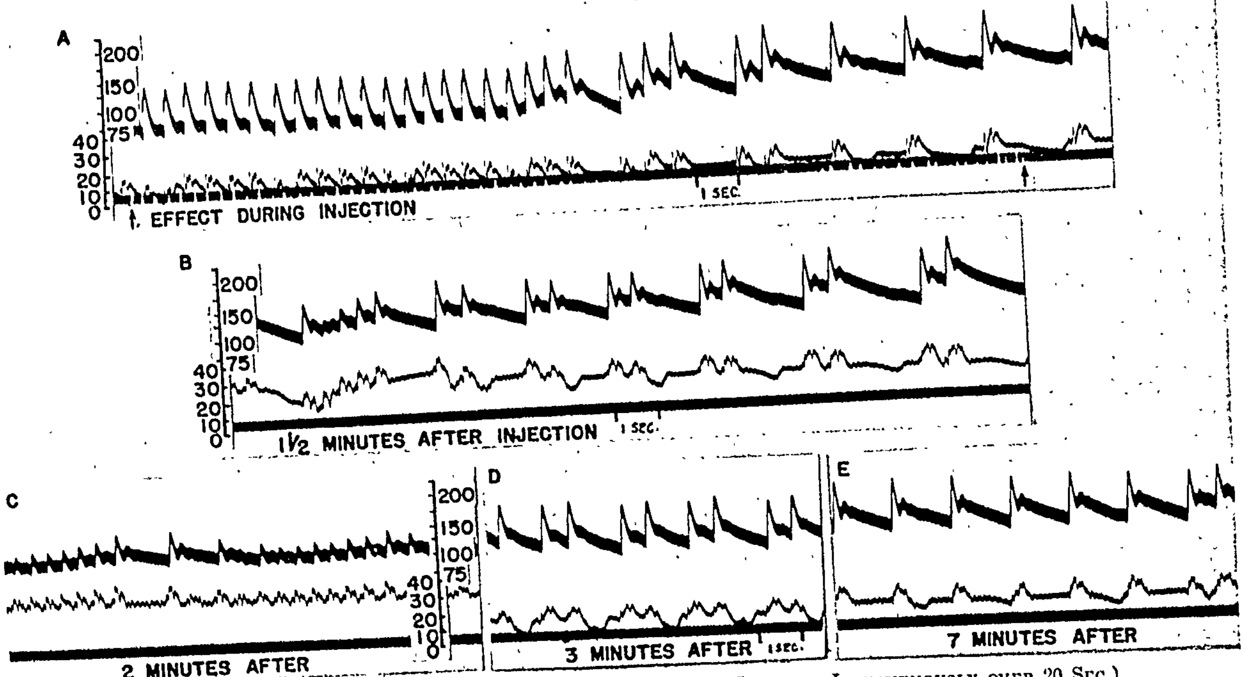

FIG. 4. A TYPICAL RESPONSE TO PITRESSIN (10 PRESSOR UNITS INJECTED INTRAVENOUSLY OVER 20 SEC.)

A is the control before, effect during (marked by two arrows) and immediately after the injection. *B*, *C*, *D* and *E* were taken 1½, 2, 3 and 7 minutes respectively after the injection. Calibration as in fig. 1. Time is indicated below. Upper curve is femoral arterial pressure; lower curve, pulmonary arterial pressure. The slight distortions by vibrations of the latter curve is on the same basis as described in the previous figure. Discussed in text.

pitressin[3] slowly intravenously over 20 to 25 seconds. Two typical experiments are shown in figures 4 and 5. Usually pulmonary arterial pressure rose, and this rise paralleled the decrease in systemic pressure pulse (fig. 4). Only in two experiments, of which figure 5 is one example, was any fall in pulmonary pressure noted. In both the drop was temporary and preliminary to the later rise in pressure (compare A and B of figure 5). In the experiment illustrated,

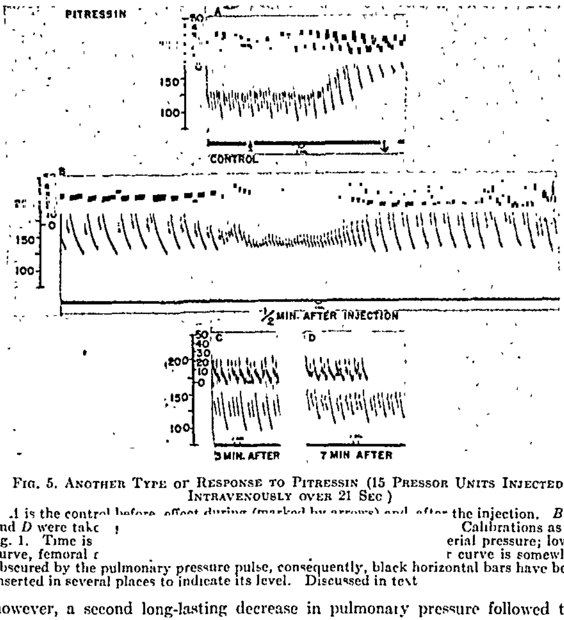

FIG. 5. ANOTHER TYPE OF RESPONSE TO PITRESSIN (15 PRESSOR UNITS INJECTED INTRAVENOUSLY OVER 21 SEC.)

A is the control before, effect during (marked by arrows) and after the injection. B, C and D were take [illegible] Calibrations as in fig. 1. Time is [illegible] erial pressure; lower curve, femoral [illegible] r curve is somewhat obscured by the pulmonary pressure pulse, consequently, black horizontal bars have been inserted in several places to indicate its level. Discussed in text

however, a second long-lasting decrease in pulmonary pressure followed the temporary pressure rise.

Our results on the *systemic* arterial pressure with this pressor fraction of the posterior lobe of the pituitary are different from those reported by Gruber (30) and Melville (31) on unanesthetized dogs. The former reported that 0.3 to 0.5 cc. of this fraction caused a drop in systemic pressure and the latter also reported a drop in systemic blood pressure using doses of 1 pressor unit per kilogram. Their results differed from previous observations on anesthetized

[3] Generously supplied by Parke, Davis and Company

animals (32) in which only a systemic pressor effect was noted. In the doses we used we obtained only a long continued pressure elevation in all seven experiments in the unanesthetized dog. Only when temporary tachycardia developed (figs. 4 and 5) did a dip occur but even then the pressure remained above normal. The pressure rise in the systemic arteries in our unanesthetized trained dogs was long lasting (about 10 to 15 minutes or more), and so were the effects on pulmonary pressure. These results on systemic pressure agree with those reported by Grollman and Geiling (33) on man following the intramuscular injection of 8 units of the drug.

Like Grollman and Geiling we consistently found a decrease in pulse rate (figs. 4 and 5) and despite the slowing, a decline in systemic pulse pressure (fig. 5). We attribute this decrease in pulse pressure to a depression of the heart following intense coronary vasoconstriction (28). A decrease in cardiac output and stroke output, has been reported in anesthetized animals (77) and in man (33, 34). The sinus slowing was accompanied in our experiments by marked sinus arrhythmia (figs. 4 and 5) and by periods of rapid heart action, presumably runs of premature beats, during which the decrease in pulse pressure was extreme (fig. 4).

The pulmonary arterial pulse also showed a decline in amplitude especially during the periods when the pressure level fell (fig. 5). Apparently, the slowing in rate tended to neutralize this, and at times as in fig. 4, actually led to a slight increase in pulse pressure.

A comparison of figs. 4 and 5, we believe, reveals the clue to the changes in pulmonary arterial pressure. Thus, in fig. 4 when the pulmonary pressure rose, it will be seen that while the systemic pressure pulse decreased, the pulmonary pulse increased (compare A and B). This we take to indicate that the decline in the contractile power of the heart was greatest in the left ventricle and that therefore we are dealing with left heart failure and consequent pulmonary congestion, which is aggravated (fig. 4) when the heart rate is accelerated. In fig. 5, on the contrary, in the periods when the pulmonary pressure rose, the pulmonary pressure pulse declined despite the slowing while the systemic pressure pulse showed little change (compare A and latter part of B). This we take to indicate a greater decline in the contractile power of the right than the left ventricle with a consequent decrease in pulmonary congestion. The reversal in the relative contractile power of the two ventricles at the beginning of segment B, appears to account for the temporary rise in pulmonary pressure.

While other mechanisms may contribute to these pulmonary pressure changes, notably redistribution of blood and pulmonary congestion consequent on shrinkage of the systemic vascular bed, they probably play an unimportant rôle in comparison with the direct depressant effect of pitressin on the heart, presumably the result of its extreme coronary vasoconstrictor action. These experiments therefore illustrate the dependence of the pulmonary pressure on the relative contractile power of the two ventricles. Pulmonary pressure will rise whenever the right ventricle is more powerful than the left because of increased venous return, or when it suffers the ill effects of drug or other depression less

than the left. On the other hand, pulmonary pressure will fall whenever the right ventricle is weaker than the left because of a decreased venous return or because it is the more depressed.

It would appear that the vasomotor action of pitressin on the pulmonary vessels is insignificant by comparison with these other indirect effects.

Paredrine. No previous observations have been made, as far as we could ascertain, on the effects of paredrine on the pulmonary pressure. It has been established both in animals (35) and man (25) that this drug has a systemic pressor effect of long duration. It does not appear to affect cardiac output, pulmonary circulation time, circulating blood volume or vital capacity in man (25) and it is a definite coronary vasodilator (14).

Paredrine was tested six times in 4 trained unanesthetized dogs in doses ranging from 2 to 20 mgm.; the paredrine hydrobromide[4] was injected intravenously over 1 to 5 sec. depending on the dose. A typical experiment is shown in fig. 6. Our results in the unanesthetized trained dog agree with those in the anesthetized dog and in man regarding systemic blood pressure and heart rate. These effects were similar to those of adrenalin except that they were more prolonged, lasting up to 1 hour or more, and the heart rate changes were not as marked. The systemic pulse pressure also increased, probably because of the elasticity coefficient in the arterial vessels under the higher pressure level. The slowing in heart rate with its associated increase stroke output would contribute to increased pulse pressure.

The pulmonary arterial pressure in all experiments rose definitely and remained elevated during the entire period of the systemic pressor effect. In 2 of the 6 experiments it was found that the pulmonary arterial pressure actually began to rise before the systemic (fig. 6). The rise in diastolic pressure was marked, averaging 12 mm. Hg and sometimes increasing as much as four-fold. The pulse pressure rose even more, averaging 16 mm. Hg and in one case equaling 35 mm. Hg; the increase in the pulmonary pressure pulse was caused by mechanisms similar to those outlined above for the systemic pressure pulse rise.

In the case of paredrine the pulmonary arterial pressure rise cannot be attributed to a redistribution of blood or to left heart failure since no evidence of pulmonary congestion has been found in man (36) and the evidence in man and animals fails to reveal any depression of cardiac action or any coronary vasoconstriction. In fact the evidence is just the reverse as regards these two functions (14, 76). The large increase in pulmonary arterial pressure without pulmonary congestion therefore suggests that this drug has a marked pulmonary vasoconstrictor action just as it has on the systemic vessels (excluding the coronary vessels). The lack of appreciable lag of the pulmonary pressure rise in relation to the systemic and its actual precedence over the latter favor this interpretation. This presumption of pulmonary vasoconstriction should, however, be tested directly.

The presence of pulmonary arterial hypertension without an associated pul-

[4] We are indebted to Smith, Kline and French Laboratories who supplied us with the needed material.

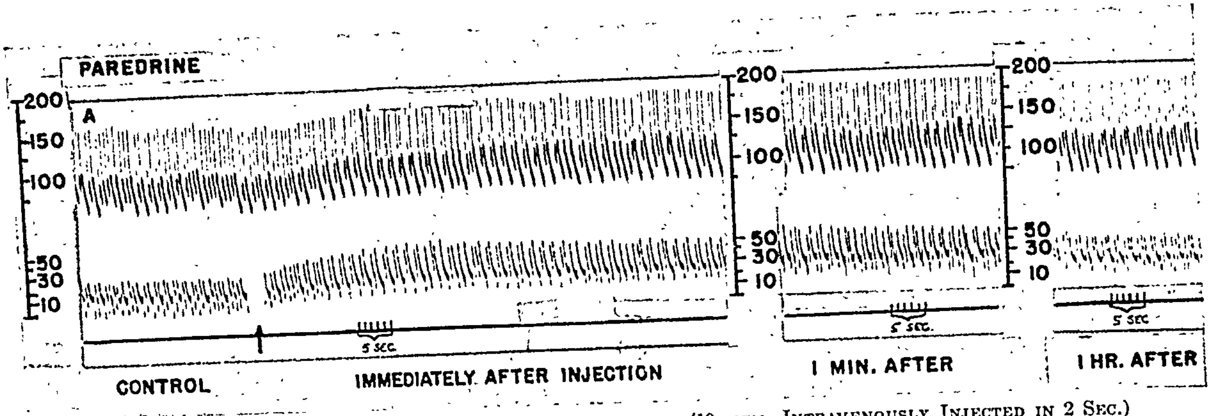

FIG. 6. A TYPICAL RESPONSE TO PAREDRINE HYDROBROMIDE (10 MGM. INTRAVENOUSLY INJECTED IN 2 SEC.)

A is the control before, effect during (at arrow) and immediately after injection. *B* and *C* were taken respectively 1 min. and 1 hour after injection. Calibrations as in fig. 1. Time is indicated below. Upper curve is femoral arterial pressure; lower curve, pulmonary arterial pressure. The distortion of the latter curve is on the basis explained in Fig. 3 and the comment on the reliability of the record there stated holds for this record also. Discussed in text.

monary congestion emphasizes that the two terms are not necessarily synonymous and that congestion occurs only when the engorgement extends to the smaller peripheral pulmonary vessels and veins, and is not confined to the arteries. The absence of demonstrable pulmonary congestion with this relatively slowly acting pressor drug shows that the degree of congestion in the lung is dependent on the rate at which the peripheral systemic vessels are contracted. In slow-acting pressor drugs apparently systemic depots are capable of being utilized to a greater extent than with quick-acting ones. It would thus appear that paredrine is better suited than other pressor drugs to elevate systemic pressure without the accompanying ill effects of pulmonary congestion. However, it does lead to a marked and sustained pulmonary hypertension which may be detrimental in a patient with a weak right heart.

Digitalis. In the anesthetized dog the effect of digitalis is to increase the pulmonary arterial pressure (37). The pulmonary pressure elevation was attributed by Wiggers (38) to an active vasoconstriction of the pulmonary vessels since it was unaccompanied by elevation of pulmonary *venous* pressure. In reviewing the experimental data, it was concluded by Weese (39) that the pulmonary vascular effects occurred only with massive doses and not with therapeutic doses of the drug.

We have investigated the action of digitalis in 4 experiments on 3 unanesthetized trained dogs. In all digifoline* was used. In 2 of these, 5½ cat units was injected intravenously and in the other 2, the dose was ½ cat unit. In none of these experiments was there any indication of a significant change in pulmonary arterial pressure. A typical experiment with the larger dose of the drug is shown in fig. 7. In all the well-known pressor effect on the systemic arterial pressure of the dog (40) was noted.

It would appear, therefore, that in the unanesthetized trained animal, any pulmonary vasomotor effect is so slight that the decrease in venous return and circulating blood volume which digitalis is known to produce in the dog (40), and in man (41), is sufficient to prevent pulmonary arterial hypertension. There is, therefore, no reason to expect on the basis of our experiments, that digitalis will have any great influence on pulmonary arterial pressure in normal man. Obviously, however, these results may not apply when pulmonary congestion, such as occurs in heart failure, is present.

Acetylcholine. Bennati *et al.* (42) found that 0.1 mgm. in the chloralosanized open-chested dog caused a pulmonary arterial pressure rise while Johnson *et al.* (17) found that 40 mgm. in the lightly etherized closed-chested dog caused a drop in pulmonary arterial pressure. In the present study, 3 experiments were done on 3 trained unanesthetized dogs using 0.025 to 0.25 mgm. acetylcholine[5] intravenously. In two experiments, no pulmonary arterial pressure change occurred, in the third, shown in fig. 8, a rise in pulmonary diastolic pressure, without change in the systolic, occurred at the height of the marked systemic depressor effect.

* Generously supplied by Ciba Company.

[5] Generously supplied by Hoffmann-LaRoche.

A correlation of this data with the reports previously published reveals that the change in pulmonary pressure is related to the change in heart rate. Thus, in the experiment illustrated (fig. 8) there was marked cardiac acceleration accompanying the pulmonary pressure rise. In Bennati's experiments (42), the heart rate also increased. In the other two experiments in the present study, no significant change in pulse rate occurred. In the experiments of Johnson *et al.* (17) the drop in pulmonary arterial pressure occurred with an extreme

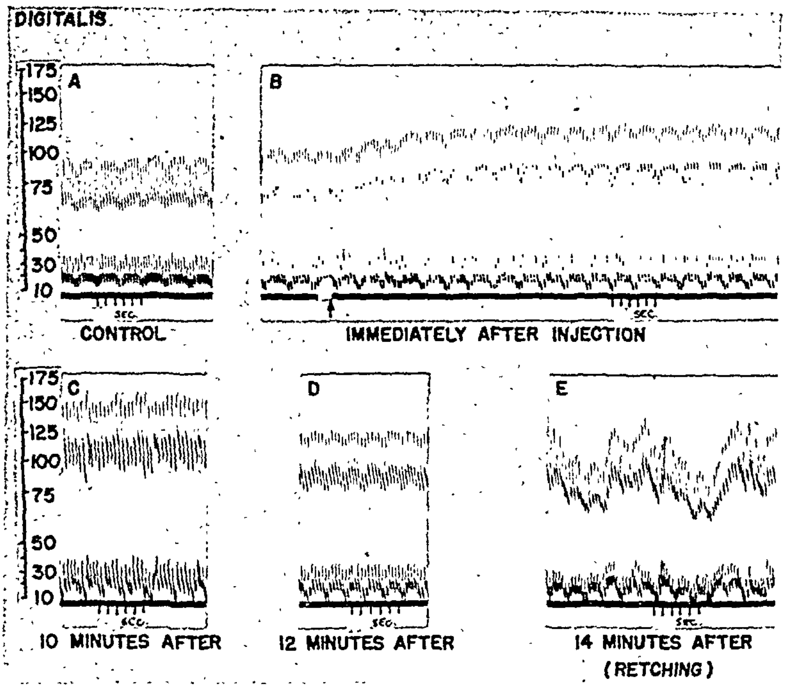

FIG. 7. A TYPICAL RESPONSE TO A LARGE DOSE OF DIGITALIS (11 CC. OF DIGIFOLINE INJECTED INTRAVENOUSLY OVER 2 SEC.)

A is the control record; *B*, immediately before, during (at arrow) and after injection; *C*, *D* and *E*, taken 10, 12 and 14 minutes respectively after injection. The effect on the pressures of retching induced by digitalis is shown in *E*. Calibration as in fig. 1. Time is indicated below. Femoral arterial pressure is above, pulmonary arterial pressure below. Discussed in text.

slowing of the heart rate. It is obvious that when the heart slows as much as shown in fig. 5B of the report of Johnson *et al.*, blood will run off into the veins faster than it enters the arteries and this will lead to a drop in arterial pressure, pulmonary as well as systemic. On the other hand, with the decrease in output of the left heart accompanying such slowing, the pulmonary venous pressure would be expected to rise, as was observed by Johson *et al.* (17).

Conversely, several factors operate to increase the pulmonary arterial pres-

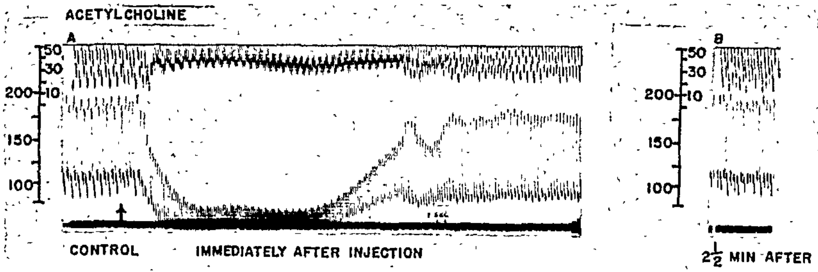

FIG. 8. AN EXPERIMENT TO SHOW ONE EFFECT OBTAINED WITH ACETYLCHOLINE (0.05 MGM. INJECTED INTRAVENOUSLY OVER 1 SEC.)

A is the control before, effect during (at arrow) and after injection. *B* was taken 2½ minutes after the injection. Calibrations as in fig 1. Time is indicated below. Pulmonary arterial pressure is above, femoral arterial pressure below. Discussed in text.

sure when tachycardia and a marked systemic depressor effect occur. One is left heart failure which develops because the rapid heart action increases the energy consumption of the heart at the same time that the marked drop in systemic pressure decreases coronary blood flow even though acetylcholine dilates the coronary vessels in the dog (28). This left heart failure produces pulmonary congestion. At the same time, the dilatation of the peripheral systemic vessels might be expected to facilitate the venous return to the right heart temporarily and so increase the amount of blood pumped into the pulmonary vascular system.

In short, the pulmonary pressure rise observed in one of our experiments is attributable to a disparity in the pumping action of the two ventricles. The absence of such disparity in the others would rule out this case of pulmonary pressure change.

Pulmonary vasodilation with small doses of acetylcholine and pulmonary constriction with large doses (43, 44) seem therefore to play an unimportant rôle in varying the pulmonary pressure and the effects appear instead to be secondary to these indirect actions of the drug. The depressor effect on systemic blood pressure and the secondary reflex acceleration (Marey effect) is easily elicited in the unanesthetized trained dog. We have been compelled to use small doses since we have found in preliminary experiments that larger doses led to such marked and protracted hypotensions that alarming reactions occurred. These are not so apparent in anesthetized animals. In man, the action of acetylcholine seems less powerful. Ellis and Weiss (45) found that continuous injections of 20–60 mgm./min. intravenously were required to produce a drop in systemic arterial pressure. On this account it would appear from our results that little or no change in pulmonary arterial pressure would be expected in man with this drug.

Papaverine. Observations on cupaverin, pharmacologically similar to papaverine, indicate that it produces a fall in systemic arterial pressure in the anesthetized cat, and an accompanying pulmonary arterial pressure elevation (46). The action of papaverine on the pulmonary vascular system has been tested in pulmonary embolism (47, 48) in order to evaluate its mode of action in this condition for which it has been clinically recommended (49). It was found that both in the anesthetized and unanesthetized animal, papaverine lessened the pulmonary congestion caused by pulmonary embolism as revealed by a decrease in the size of the vascular tree visualized with diodrast (47, 48). At the same time the right heart decreased in size and the pulmonary diastolic arterial pressure was reduced towards normal (47). The effect of papaverine was greatest when the smallest vessels were occluded and least when the main branch was plugged (47).

It was the purpose of the present study to determine whether a similar reduction in the pulmonary vascular bed as evidenced by a decrease in pulmonary pressure occurred in the unanesthetized trained normal dog. Six experiments done on 2 different dogs, using 32 to 64 mgm. of the papaverine hydrochloride[6]

[6] Kindly supplied by Dr. K. K. Chen of Eli Lilly Co.

injected intravenously over 3.5 sec., gave consistent results. A typical experiment is shown in fig. 9. A definite transitory drop in systemic pressure occurred accompanied by a noticeable tachycardia; this is well established (50). Following this depressor effect the pressure level and pulse pressure sometimes showed a slight overswing beyond normal (fig. 9).

The pulmonary arterial pressure rose (fig. 9) and as the temporary tachycardia lessened, the pulse pressure also increased, sometimes by as much as 100 per cent. These pulmonary arterial pressure changes outlasted the effect on the systemic vessels and sometimes persisted for several minutes.

Apparently the effects of papaverine on the pulmonary arterial pressure are different in the presence of pulmonary embolism than in its absence, except that under both circumstances the pressure pulse increased. There is as yet insufficient data on the action of papaverine on the pulmonary vasculature and cardiac output to account for these results. Nevertheless it would appear that papaverine does dilate the pulmonary vessels as well as the systemic (50) and coronary vessels (51, 52, 53) and that this pulmonary vasodilation dominates the result when pulmonary congestion occurs and possibly when pulmonary embolism of the smaller vessels develops. In the normal dog, however, this action is counterbalanced by another indirect action, apparently a stimulation of the heart whereby the stroke volume increases and with it the pulse pressure despite cardiac acceleration. The peripheral dilatation of the systemic vessels with perhaps a decrease in the capacity of the systemic blood reservoirs, causes an increase in venous return to the right heart and a surge of blood to the lungs which serves to elevate the pressure level and pressure pulse for some time.

These results with papaverine indicate the need for further investigation on the mode of action of this drug.

Histamine. The action of this drug on the pulmonary circulation has been extensively studied but the results are not entirely in accord. It has also become apparent that the effects in one species are not always identical in net result or *modus operandi* to those of other species (54).

Constriction of isolated strips of pulmonary arteries have been reported in the rabbit (55), cat (56) and other species (54); in the dog, however, dilatation follows the use of histamine (57). In perfused lungs, pulmonary vasoconstriction occurs after administration of histamine in the cat (43, 56) monkey (58) and dog (59).

In the heart-lung preparation of the dog, histamine causes pulmonary hypertension (60, 61). A similar effect is obtained in the decerebrated guinea pig (62), in the anesthetized rabbit (63, 64), cat (61, 64) and dog (63, 64). However, in the anesthetized dog the rise is slight and transient and is followed by a more protracted fall (63, 64).

The results in the unanesthetized dog are different. Thus, Woodbury and Hamilton (64) found in a series of experiments on two dogs using intravenous histamine in doses of from 0.4 to 2 mgm. that only a temporary pulmonary pressure rise occurred after the systemic pressure had begun to fall.

We repeated these observations in four experiments on three unanesthetized

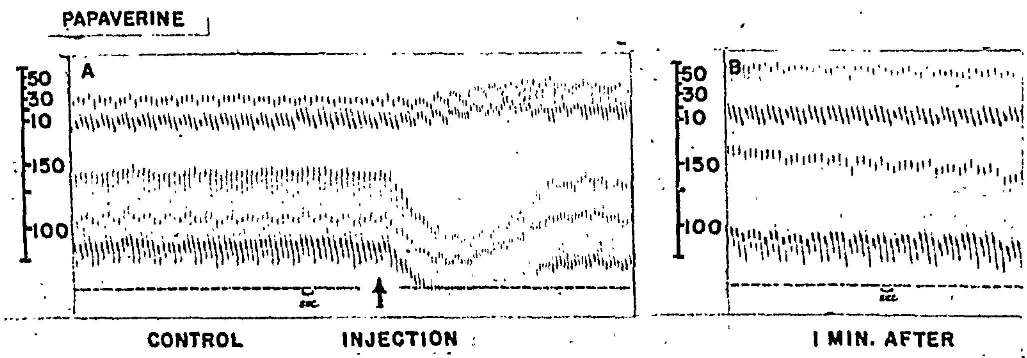

Fig. 9. A Typical Experiment Showing the Effect of Intravenous Injection of 61 mgm. of Papaverine Hydrochloride over 3 Sec.

A is the control before, effect during (at arrow) and immediately after injection. *B* was taken 1 minute later. Calibrations as in fig. 1. Time is indicated below. Pulmonary arterial pressure is above, *femoral arterial pressure* below. Discussed in text.

trained dogs using 0.3 to 2 mgm. of histamine acid phosphate[7] intravenously injected slowly over 1 to 45 seconds. A typical experiment is shown in fig. 10. Our results agree with those of Woodbury and Hamilton in that we obtained a rise in pulmonary pressure which appeared only after the systemic pressure had begun to fall. However, in our experiments, the pulmonary arterial pressure changes persisted for several minutes after the systemic pressure had returned to normal. This is similar to the effects with papaverine observed by us (see above).

In our experiments the rise in pulmonary pressure was manifested by elevation of systolic pressure. In three experiments the diastolic pressure remained unchanged, and only in the one illustrated in fig. 10 did it rise. In all four experiments the pulse pressure rose, and this without heart slowing, or in spite of cardiac acceleration (fig. 10).

We are in accord with Woodbury and Hamilton (64) that the effect on the pulmonary circuit is an indirect one and not due to pulmonary vasoconstriction. The absence of diastolic pressure elevation in 3 of our four experiments and the observation of Dixon and Hoyle (63) that an increase in pulmonary venous outflow accompanies the pulmonary pressure rise in the anesthetized dog favors this interpretation.

Apparently the effect of histamine is due to an increased venous return and cardiac output which causes the right heart to pump blood into the pulmonary circuit at an increased rate thereby elevating its pressure. Such an increase in cardiac output has been found in animals (61) and man (65) and accounts for the increase in pressure pulse in our experiments. This increase in cardiac output is associated with coronary dilatation (53). The effect on the heart and pulmonary vascular system outlasts the well known (19) systemic depressor effect which can be more quickly readjusted by the buffer nerve mechanism.

Amyl Nitrite. It is established that the nitrites are direct dilators of smooth muscle of the vascular tree (19) including the coronary vessels (28, 51) and would be expected to lead to a depressor action on the systemic arterial blood pressure. This is the effect observed in anesthetized animals (66). However, in the unanesthetized animal, amyl nitrite has been shown to cause consistently a rise in systemic arterial pressure (67), a fact confirmed in our own studies.

The difference in the results in anesthetized and unanesthetized animals might possibly result in different effects on the lesser circuit. In the isolated pulmonary vessels conflicting reports have appeared; constriction (75) and dilatation (68) both being reported. In the anesthetized dog a rise in pulmonary arterial pressure was reported with amyl nitrite inhalation (69). In the unanesthetized dog, however, only a slight rise in pulmonary pressure has been found (18).

We repeated the experiments with amyl nitrite inhalation in 5 trials on four trained unanesthetized dogs and obtained consistent results. One to three ampules of amyl nitrite[8] (0.18 cc. each) were inhaled over a period of from 2 to

[7] Generously supplied by Eli Lilly and Co.

[8] Generously supplied by Eli Lilly and Company.

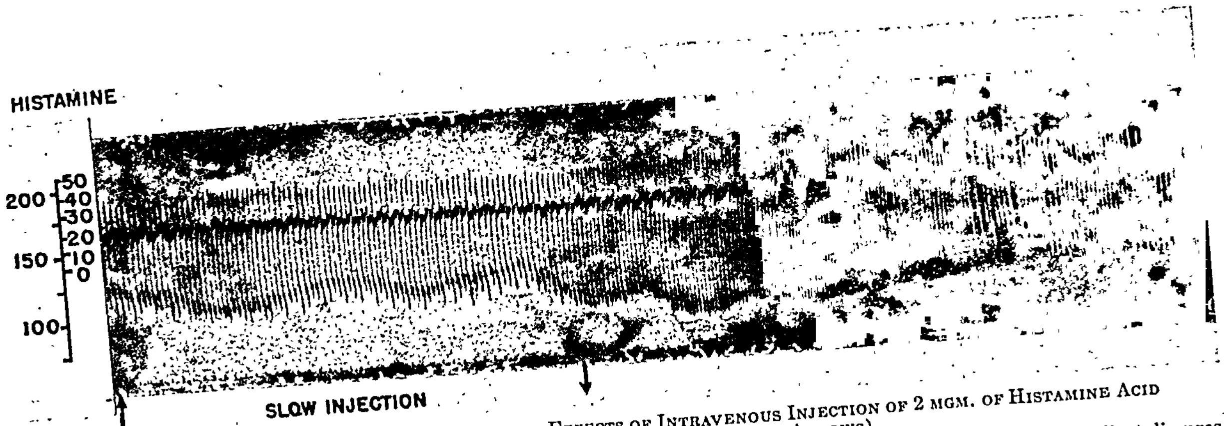

FIG. 10. AN EXPERIMENT SHOWING THE IMMEDIATE EFFECTS OF INTRAVENOUS INJECTION OF 2 MGM. OF HISTAMINE ACID PHOSPHATE OVER 45 SEC. (AS INDICATED BY ARROWS)

This experiment differs from the others with this drug which lacked the temporary tachycardia and the rise in diastolic pressure but was similar in other respects. Calibrations as in fig. 1. Time is indicated below. Pulmonary arterial pressure is above, femoral arterial pressure below. Discussed in text. It is regrettable that the record was spoiled in the process of being developed, but the pressure pulses can be made out clearly.

70 secs. In all the pulmonary diastolic pressure was unchanged and only in one, fig. 11, in which the most noticeable slowing of the heart occurred was the systolic pulmonary pressure raised. In the others, in which heart rate was unchanged or only slightly slowed, no change in systolic pressure occurred. In all, as in fig. 11, respiration was deepened and the respiratory fluctuation in pulmonary pressure increased. Our results, therefore, in accord with Hamilton *et al.* (18), show that in the unanesthetized dog the pulmonary arterial pressure does not rise with amyl nitrite.

It would appear that several opposing factors operate in the unanesthetized animal. The evidence is clear that amyl nitrite increases cardiac minute output in unanesthetized animals (70) and in man (quoted from 21), and this apparently is accompanied by systemic vasoconstriction which is apparently due to a reflex from irritation of the air passages (67) involving higher centers at the conscious level and which may perhaps involve the disturbing reaction to a strange odor. The output of the two ventricles appears to remain in balance so that no pulmonary congestion occurs in the unanesthetized animal. However, even if the effect were greater on the right heart, pulmonary vasodilation by increasing the capacity of the circuit would permit the accomodation of the increased quantity of blood coming to the lungs with little or no pulmonary pressure rise.

These cardiodynamic results with amyl nitrite show clearly how anesthesia may alter drug action. This again should emphasize the need of reinvestigating drug actions in the absence of anesthesia if the results of animal experimentation are to be more usefully applied to man.

Aminophyllin. Aminophyllin (theophylline ethylinediamine) has been used clinically not only as a coronary dilator but for the relief of Cheyne-Stokes respiration (71) and paroxysmal dyspnea especially the bronchial variety (72). In man it has been found to have little effect on systemic arterial pressure (21), to decrease systemic venous pressure (72), to increase cardiac output, to accelerate the heart and to increase the depth of respiration (21).

In anesthetized dogs, it also causes a fall in systemic arterial pressure and cardiac acceleration (73, 74). It has a coronary dilator action as well (51, 53). No work has appeared as far as we know on its effects on the pulmonary circulation nor has its systemic action on the unanesthetized animal been investigated.

We have tested aminophyllin[9] four times in 3 unanesthetized trained dogs using 0.12 to 0.24 grams of the drug injected intravenously over 2.5 to 30 seconds. Fig. 12 is a typical experiment. The effect of aminophyllin in enhancing respiration was slight. In all cases there was a slight cardiac acceleration, and a slight temporary fall in systemic arterial pressure with an increase in pulse pressure which persisted for some time after the diastolic pressure had returned to normal.

The pulmonary diastolic pressure rose as the systemic arterial pressure fell in one experiment (fig. 12). In the others the pulmonary diastolic pressure did not change. The pulmonary systolic and pulse pressure rose in all cases. The rise in the pulmonary pressure pulse in the experiment shown in fig. 12 lagged behind

[9] Generously supplied by Dubin Laboratories.

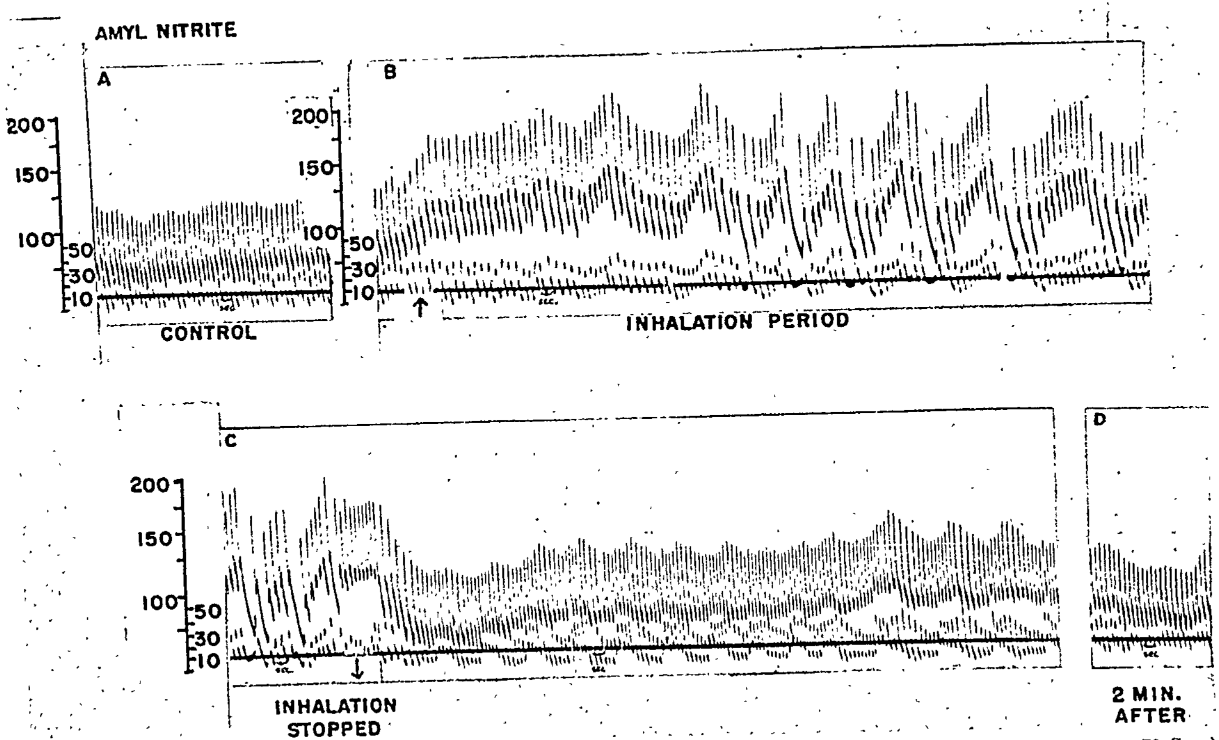

FIG. 11. AN EXPERIMENT SHOWING THE EFFECT OF INHALATION OF AMYL NITRITE (3 AMPULES (EACH 0.18 CC.) OVER 70 SEC.)
A is the control; *B*, the period of inhalation (begun at arrow); *C*, end of inhalation (indicated by arrow) and immediately after; *D*, 2 minutes after end of inhalation. Calibration as in fig. 1. Time is indicated below. Femoral arterial pressure is above, pulmonary arterial pressure below. Discussed in text.

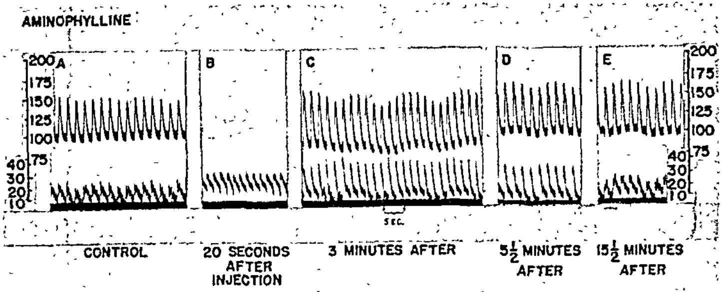

FIG. 12. AN EXPERIMENT SHOWING EFFECT OF AMINOPHYLLIN (0.12 MGM. INJECTED INTRAVENOUSLY OVER 2 5 SEC.)

A is the control. *B*, *C*, *D* and *E* were taken respectively 20 seconds, 3, 5½ and 15½ min. after the injection. Calibrations as in fig. 1. Time is indicated below. Femoral arterial pressure is above, pulmonary arterial pressure below. In *A* and *E* there is an artefact in the pulmonary arterial curve which does not prevent pressure measurements. Its cause has been discussed in legend of fig. 3. In *B*, the femoral pressure curve is blocked out since the needle was clotted. The clot was removed between *B* and *C*. Discussed in text.

the diastolic pressure rise, and continued to increase after the diastolic pressure had returned to normal.

The increased pulse pressure following aminophyllin in both pulmonary and systemic circuits suggests an increase in cardiac stroke volume occurring despite cardiac acceleration, indicating an increased minute volume output. It cannot be attributed to the elastic coefficient of the vascular tree as with pressor drugs. The temporary rise in pulmonary diastolic pressure in one experiment may indicate a greater effect on the right heart than on the left. The early restoration of the pulmonary diastolic pressure to normal in this case may indicate either the counter effect of pulmonary vasodilation, which has not yet been demonstrated, or more likely a readjustment of the outputs of the right and left ventricles.

The fact that aminophyllin leads to a sustained elevation of the mean pressure in this circuit indicates that its beneficial action clinically is not due to relief of pulmonary congestion. However, this statement requires further investigation in conditions with pulmonary congestion since it is possible that, as in the case of papaverine (see above), it may have an opposite effect when pulmonary congestion is present. Until this is investigated, however, we must conclude that the beneficial action of aminophyllin is on the respiratory center, perhaps by dilating the bronchioles, or it may have a direct stimulating action on the heart.

SUMMARY AND CONCLUSIONS

1. A method is described for obtaining simultaneously accurately recorded pulmonary and systemic arterial pressure pulses under the influence of various drugs in the trained unanesthetized dog.

2. The factors which may be contributory to effect changes in pulmonary pressure are discussed. These are in part *passive* factors, viz. *1*) mechanical effects of respiration, *2*) the relative contractile strength of the two ventricles, and *3*) large changes in circulating blood volume leading to redistribution of blood between the lungs and the systemic circuits. In part, these are *active* effects on the pulmonary blood vessels, viz. *1*) secondary to chemical changes in the blood, *2*) reflexogenic following pressure changes in the systemic circuit, and *3*) primarily due to direct action on the caliber of the pulmonary vasculature.

3. Analysis of results with eleven different drugs shows that renin, angiotonin, adrenalin, pitressin (usually), paredrine, acetylcholine (usually), histamine, and aminophyllin cause an elevation of pulmonary arterial pressure, while amyl nitrite and digitalis produced little if any change in pulmonary pressure. The modes of action of these various drugs are discussed. Of the drugs tested, only paredrine appears to produce its effect by direct vasomotor action on the lung blood vessels.

4. Experiments on trained unanesthetized animals can give information of the pulmonary and systemic circuits more applicable to man than studies on anesthetized, open chested animals, or on heart-lung preparations.

We are grateful to Dr. S. Rodbard for his assistance in conducting these experiments.

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THE PHARMACOLOGY OF N-SUBSTITUTED CARBAMINO-CHOLINES[1]

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In a series of communications Hunt and Renshaw (1) studied the pharmacologic activity of a number of choline and related ammonium compounds. The chief characteristics of these substances were their autonomic-like effects. Some of the compounds exhibited vaso-depressor (muscarinic) effects, others exhibited vaso-pressor (nicotinic) phenomena, and still others showed combined muscarine and nicotine effects. Their work consisted mostly of blood pressure studies in cats. In this paper the pharmacologic properties of a new group of *N*-substituted carbamino cholines synthesized by Sprinson (2) are reported.[3] Each of these compounds (table 1) was subjected to a number of biologic experiments.

METHOD. Pharmacologic tests were made on: (*A*) blood pressure (B.P.) recorded from the femoral artery of cat, dog, monkey and rabbit, (*B*) denervated smooth muscle: iris of cat, monkey, rabbit and dog, (*C*) denervated striated muscle such as facial muscles of monkey and cat, (*D*) isolated smooth muscle: intestines of rabbit and guinea pig and (*E*) isolated striated muscle: frog's rectus abdominus.

The iris was denervated by sectioning aseptically all the ciliary and optic nerves behind the globe. Five to eight days postoperatively this iris became sensitive to cholinergic and adrenergic substances. The sensitivity of the completely denervated (*C.D.*) iris to drugs varied with the species (3); in the cat intravenous injections of 0.01 micrograms (μgm.) of adrenalin per kilo dilated the pupil while 0.5 μgm. of doryl (carbamino choline) per kilo constricted it. The denervated facial muscles in monkeys and cats were prepared by sectioning or avulsing the facial nerve. These muscles became sensitive to acetylcholine, carbamino choline (doryl), nicotine and the compounds under consideration within 3 to 14 days after the denervation. In several cats and monkeys combined facial and iris denervations were made; usually the face was denervated on the left, and the iris on the right. Except for a few experiments performed on unanesthetized cats and monkeys, most of the observations were made under light nembutal anesthesia.

RESULTS. (*A*) *Blood pressures.* Four cats, 2 rabbits, 3 monkeys and 2 dogs were used. As a rule intravenous injection of compounds 1 to 7 listed in table 1 resulted in an initial transient fall in blood pressure. A drop of 10 mm. Hg occurred within the circulation time. Phenyl doryl (*N*-phenyl carbamino choline) was without effect. In all instances the initial drop was followed by an abrupt and prolonged rise, sometimes to 150 mm. over the original level. The secondary or delayed rise was observed in all of the four species and the degree

[1] This work has been partly aided by the Josiah Macy Jr. Foundation.

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[3] The synthesis of these compounds was carried out by one of us (D. B. S.) while a student of the late Professor R. R. Renshaw.

of elevation varied with the dose and the chemical injected. The rise in blood pressure was most conspicuous in the cat.

The primary fall in blood pressure was not a consistent or conspicuous finding. It could be abolished by atropine, which indicates that this was a muscarinic effect. The delayed rise in blood pressure must have been due to secondary influences of the drug and increased secretion of adrenaline seemed a probable explanation. Bilateral adrenalectomy or sectioning of the spinal cord diminished or abolished the delayed increase in blood pressure produced by small doses, but, larger doses were still effective in producing a delayed rise in blood pressure. This points to a peripheral action. In the cat this peripheral action was very conspicuous with doses of 0.15 mgm. per kilo of body weight.

(*B*) *Denervated iris.* Seventeen cats, 5 monkeys, 3 rabbits and 1 dog were studied. In none of these animals did compounds 1 to 8 produce constriction

TABLE 1

Minimal doses in micrograms of choline compounds injected intravenously necessary to produce contraction in denervated facial muscles of a monkey

SUBSTANCE	FORMULA	DOSE
		µgm./kilo
(1) Methyl carbamino choline	$CH_3NHCO_2C_2H_4N(CH_3)_3Cl$	25
(2) Dimethyl carbamino choline	$(CH_3)_2NCO_2C_2H_4N(CH_3)_3Cl$	40
(3) Ethyl carbamino choline	$C_2H_5NHCO_2C_2H_4N(CH_3)_3Cl$	180
(4) Diethyl carbamino choline	$(C_2H_5)_2NCO_2C_2H_4N(CH_3)_3Cl$	200
(5) Propyl carbamino choline	N—$C_3H_7NHCO_2C_2H_4N(CH_3)_3I$	25
(6) Butyl carbamino choline	N—$C_4H_9NHCO_2C_2H_4N(CH_3)_3I$	30
(7) Piperidino carbamino choline	$C_5H_{10}NCO_2C_2H_4N(CH_3)_3I$	700
(8) Phenyl carbamino choline	$C_6H_5NHCO_2C_2H_4N(CH_3)_3I$	3000*
(9) Carbamino choline	$H_2NCO_2C_2H_4N(CH_3)_3Cl$	5
(10) Acetylcholine (eserinized)	$CH_3CO_2C_2H_4N(CH_3)_3Cl$	2

* No reaction.

of the pupil whether applied locally or intravenously. However, doses of 0.5 to 2 µgm. per kilo of acetylcholine and carbamino choline caused minimal miosis when given intravenously. Larger amounts produced more marked constriction and secondary or delayed mydriasis (3). Instillation of carbamino choline into the conjunctival sac produced constriction of the pupil in all animals. On the other hand, the N-substituted compounds had no such effect. In the cat and dog all the N-substituted carbamino cholines except the phenyl derivative, when given in sufficient doses, produced a delayed mydriasis just as acetylcholine did. The mydriasis appeared from 10 to 17 seconds after intravenous injection of the substance and was simultaneous with the rise in blood pressure. The delayed mydriatic effect was not present in the monkey or rabbit. The mydriatic and vaso pressor changes were accompanied by marked pallor of the mucous membranes indicating peripheral vaso constriction. The delayed or secondary mydriasis was abolished by bilateral adrenalectomy.

However, when larger doses of the chemical were injected, the dilatation of the pupil in the *C.D.* iris recurred, but after a latency of 20 to 40 seconds. This suggested that an adrenergic substance such as sympathin (4) might have been secreted in the body of the adrenalectomized cat, which in turn acted on the sensitized dilator fibers of the iris.

(*C*) *Denervated facial muscles.* Nine monkeys, 4 cats, 2 rabbits and 1 dog were studied. In every animal tested the denervated facial muscles contracted with small doses of acetylcholine, carbamino choline and all the *N*-substituted carbamino cholines listed in table 1, except the phenyl compound. With larger doses the contraction produced by each of these drugs was marked and included all the denervated facial muscles. The contraction occurred within the circulation time and its onset coincided with the initial drop in blood pressure. The liminal dose for denervated facial muscle contraction was much less than for the initial drop in blood pressure showing that the nicotine effect was much more evident than the muscarine.

The following experiments demonstrated (*1*) the initial slight muscarine, (*2*) initial nicotine and (*3*) delayed nicotine action. The subject was a cat in which the left facial and right iris muscles were denervated two weeks previously. The blood pressure was recorded from the femoral artery under light nembutal anesthesia. (*a*) Six seconds after the intravenous injection of 90 μgm. per kilo of methyl doryl (*N*-methyl carbamino choline) contraction in the denervated left facial muscles and a fall of 10 mm. in B.P. occurred. (*b*) Twelve seconds after the injection there was dilatation of the denervated iris and an abrupt rise of 60 mm. in B.P. which lasted for more than 95 seconds. (*c*) After sectioning of the cervical spinal cord, injection of this drug produced contraction in the denervated face as before but a larger drop in B.P.; the secondary rise in B.P. was diminished; delayed mydriasis in the *C.D.* iris was present. (*d*) After bilateral adrenalectomy another injection (90 μgm.) produced the same effects as in (*a*) but no delayed mydriasis in the C.D. iris and only a slight rise in B.P. (*e*) Atropinization at this point abolished the initial vaso depression and instead a slightly delayed rise in B.P. was noted. Again there was no significant change in the pupil. (*f*) With the injection of 250 μgm. per kilo of *N*-methyl carbamino choline, there resulted a marked contraction in the denervated face, but no initial fall in B.P., a delayed but conspicuous rise in B.P. and a more delayed mydriasis in the C.D. iris. The latter effects were also observed in unatropinized preparations.

(*D*) *Isolated intestinal strips of rabbit and guinea pig.* The results obtained with isolated intestine of rabbit and guinea pig were similar. Fourteen different strips were tested in warm Locke's solution. The bath was washed after each trial. Unless high concentrations of *N*-substituted carbamino cholines were added to the perfusion bath there were no significant contractions. Whereas acetyl-choline and carbamino choline provoked good contractions in concentrations of 1×10^{-7}, none of the *N*-substituted carbamino cholines had an appreciable effect unless concentrations of 1×10^{-4} were used. This weak stimulating

action on isolated smooth muscle indicated there was relatively little muscarine effect in the drugs under investigation.

(*E*) *Isolated frog's rectus abdominus muscle.* The isolated frog's rectus abdominus was perfused with cold Ringer solution. Except for the phenyl derivative all the compounds produced a contraction of the isolated striated muscle in concentrations of 1×10^{-6} as did the same concentration of acetylcholine and carbamino choline. These reactions demonstrated the nicotinic effects of these drugs.

Discussion. Whereas carbamino choline has nicotinic and muscarinic properties the substitution of alkyl groups on nitrogen markedly reduces the muscarine activity and the substitution of a phenyl group completely abolishes both the muscarine and nicotine effects. The only evidence of a muscarinic action is the slight initial drop in blood pressure which is blocked by atropine. Other well known muscarine effects such as miosis, salivation, lacrimation, urination and defecation have not been observed. None of these compounds has any miotic action like carbamino choline itself, whether applied locally or injected intravenously in large or small doses. The absence of the miotic response and relatively weak effect on isolated smooth muscle serve as strong evidence that the compounds have relatively little muscarinic action. On the other hand, their nicotinic properties are clearly demonstrated by contraction of denervated striated muscle and stimulation of the sympathico-adrenal system. In short, the pharmacologic properties of the *N*-substituted compounds in the normal animal are almost identical with those of carbamino-choline and acetylcholine the atropinized animal.

From the temporal standpoint the actions of these drugs fall into two phases (*a*) an immediate effect, such as contraction of the denervated striated muscle, which occurs within the circulation time, and (*b*) delayed effect such as the rise in B.P. and dilatation of the pupil in the denervated iris, which occurs 4 to 10 seconds later than the circulation time. It has been shown that the delayed mydriasis is due to release of epinephrine and probably sympathin. However, the rise in B.P. is only partly due to liberated adrenergic substances. The vaso pressor effect is chiefly due to peripheral vaso constriction, for during the rise in B.P. the animal appears pale and the superficial vessels are blanched even in the adrenalectomized and spinal cat. Whether this vaso constriction is due to a stimulant action by these compounds on sympathetic ganglia, or to direct effect on the blood vessel wall, or both, cannot be concluded from these experiments. All of the *N*-substituted compounds tested act alike pharmacologically but differ in their potency. None of them is hydrolyzed by cholinesterase (5).

SUMMARY

The pharmacologic action of a group of *N*-substituted carbamino cholines was studied. These compounds show remarkable nicotinic and relatively few muscarinic properties. *N*-substitution markedly reduces the muscarine activity of carbamino choline (doryl) but the stimulating nicotine action is retained.

Illustrative protocol. Experiment Hypothal. no. 85. Cat. Weight: 3.5 kilos. Nembutal: 0.6 cc. i.v.

Time	Diameter of (right) pupil in C.D. iris	Blood pressure (systolic)	Procedures and remarks
	mm	mg. Hg	
(1)			
11/21/00	5.5	100	Methyl doryl 0.3 mgm. (90 μ g. per kilo) intravenously
11/21/06	5.5	Falling	Denervated face contracting
11/21/15		94	
11/21/18	Dilating	Rising	
11/21/22	7.5	140	
11/21/30	6.0	120	
11/22/00	5.5	100	
(2)			
12/55			Lower cervical spinal cord and roots cut completely across. This manipulation caused the right pupil to dilate to 13 mm.
(3)			
1/10/26	10.0	100	Methyl doryl 0.3 mgm. intravenously
1/10/32	10.0	Falling	Denervated face contracting
1/10/34		90	
1/10/37	Dilating	100	
1/10/43	11.0	120	
1/10/45	12.0	116	
1/11/30	10.0	100	
(4)			
1/38/00			Bilateral adrenalectomy
(5)			
2/00/00	6.2	95	Methyl doryl 0.3 mgm. intravenously
2/00/06	6 2	Falling	Denervated face contracting
2/00/15	5.8	80	Bradycardia
2/00/22		70	Slight constriction in right pupil. No secondary rise in B.P.
2/00/30	5.5	80	
2/01/00	5.5		
2/02/00	5.5		
2/05/47	5.5		
(6)			
2/15			Dimethyl doryl 0.45 mgm. intravenously had no effect on pupil of C.D. iris after bilateral adrenalectomy
(7)			Dimethyl doryl 0.6 mgm. intravenously also had no effect on pupil but delayed blood pressure rise is present
3/04/07	5.5	90	Dimethyl doryl 0 6 mgm. intravenously
3/04/15	5.5	Falling	Bradycardia. Marked contraction in denervated face
3/04/20	5.0	70	
3/04/22	5.2	60	
3/04/25	5.5	Sudden rise	No change in pupils
3/04/37	5.5	160	

ILLUSTRATIVE PROTOCOL—(*Concluded*)

TIME	DIAMETER OF (RIGHT) PUPIL IN *C.D.* IRIS	BLOOD PRESSURE (SYSTOLIC)	PROCEDURES AND REMARKS
	mm.	*mm. Hg*	
3/04/44	5.5	180	
3/04/49	5.5	190	
3/05/09	5.5	200	
3/06/45	5.5	180	Pulse rate rapid
3/07/55	5.5	150	
3/09/20	5.5	100	
3/11/17	5.5	90	
(*8*)			After atropine primary vaso-depression no longer present
3/22/27			Atropine 1 mgm. intramuscularly
(*9*)			
3/24/00	5.5	110	Methyl doryl 0.3 mgm. intravenously
3/24/08	5.5	110	Contraction in left face
3/24/12	5.5	Slight rise	
3/24/50	5.5	120	
3/25/05	5.5	110	There was no change in pupils
(*10*)			Large doses of methyl doryl produced delayed mydriasis
3/43/15	5.5	110	Dimethyl doryl 0.8 mgm. intravenously
3/43/21	5.5		Marked contraction in denervated face
3/43/24	5.5	Abrupt rise	
3/43/32	5.5	180	
3/43/40	5.5	260	
3/43/44	5.8	280	Pulse accelerated. Marked pallor of mucous membrane
3/43/55	Dilating		
3/44/06	7.5	Declining	
3/44/20	7.8		
3/44/30	7.0	220	
3/45/00		180	
3/45/55	6.5	150	
3/47/28	6.2	115	
3/47/43	6.0	110	
3/48/23	6.0	100	
3/51/12	5.5	90	
(*11*)			
3/53/29			Dimethyl doryl 0.2 mg. per kilo again produced an increase in B.P. to 240 mm. but no change in pupil. Doses above 0.3 mgm. per kilo were lethal

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PHARMACOLOGY AND CHEMISTRY OF SUBSTANCES WITH CARDIAC ACTIVITY[1]

II. Effect of *l*-Ascorbic Acid and Some Related Compounds and of Hydrogen Peroxide on the Isolated Heart of the Frog

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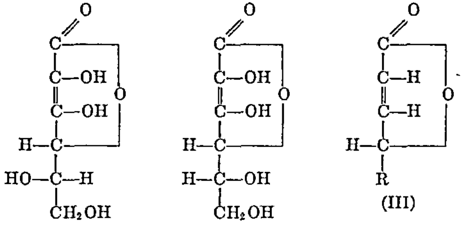
l-Ascorbic acid (I) and *d*-iso-ascorbic acid (II) can be considered as $\alpha\,\beta$-unsaturated lactones related in structure to the $\alpha\,\beta$-unsaturated lactones of type III. It therefore seemed possible that their action upon the isolated frog heart



(I) (II) (III)

might be similar to the action of the angelicalactones which we recently described (1). Urban and Peugnet (2) studied the action of *l*-ascorbic acid on the frog heart employing the technique of Straub and observed a "beat strengthening" action, an acceleration in heart rate, and, although not consistently, an increase in the "diastolic tonus." Peugnet in a further study (3) found that the presence of small amounts of copper was necessary and that the product formed when *l*-ascorbic acid was oxidized with iodine was ineffective. Preliminary experiments with our modification of Straub's technique confirmed some of the observations of Urban and Peugnet and showed that an adequate concentration of *l*-ascorbic acid administered continuously caused irreversible systolic standstill, as was observed with the angelicalactones (4). The present paper deals with the results of a more detailed study of this action and with the analysis of its cause.

Method. The experiments were carried out on frogs (mostly male) of the species *Rana pipiens* between February and May and October and November, 1942 A few preliminary experiments were conducted with the Clark solution used in our previous experiments. For

[1] This work was carried out under the auspices of the University Committee on Pharmacotherapy.

the experiments described here, however, a well-buffered solution was needed. We chose a bicarbonate buffer solution similar to that of McLean and Hastings (5), of the following composition: sodium chloride 0.5%; potassium chloride, 0.014%; calcium chloride anhydrous, 0.011%; and sodium bicarbonate, 0.23%. This solution was saturated with a gas mixture containing either 95% oxygen and 5% carbon dioxide or 95% nitrogen and 5% carbon dioxide. In order to prevent a change in the oxygen and carbon dioxide tension, rubber bags filled with the gas mixture being used were attached to the Mariotte bottles which served as reservoirs. The heart cannula had to be adapted to our special needs (see fig. 1). The fluid dropped into the wide part of the cannula at A, and, with the heart attached to the cannula, left it by way of the side tube at C. The capacity of the cannula from the level of the fluid in A to the side tube C was approximately 2.5 cc. The side tube C was so arranged that the height of the fluid column above the tip of the cannula was not more than 3 to 4 cm. The hydrostatic pressure to which the ventricle was exposed was therefore higher than 2 to 3 cm. as in Straub's original arrangement (6) but lower than in our own previous technique (1). A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the

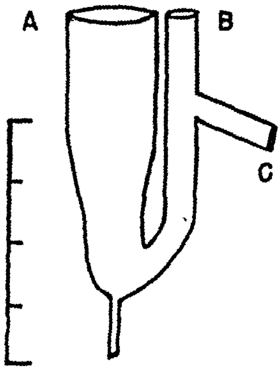


FIG. 1. CANNULA FOR ISOLATED FROG HEART (SCALE IN CM.)
In order to remove fluid from close proximity to the heart a hollow needle can be inserted through the opening at B. For further description see text.

fluid in the wide part of the cannula in order to maintain the carbon dioxide tension. Moreover, this procedure guaranteed the necessary supply of oxygen to the heart in those experiments in which the solution in the Mariotte bottle was kept under nitrogen. The rate of replacement of the solution in the cannula was 2 to 2.5 cc. per minute.

The *l*-ascorbic acid and the related compounds were dissolved in distilled water and neutralized with sodium bicarbonate, the sodium salts being used in making up the solutions. As some of the substances tested are readily destroyed while standing in a solution containing oxygen, concentration, unless otherwise specified, refers to the initial concentration of the tested agent. Repeated tests with the glass electrode indicated that the pH range of of the fluid entering the heart was between 7.5 and 7.8 in all experiments.

The *l*-ascorbic acid and the *d*-iso-ascorbic acid used were the crystalline synthetic products.[2] The dimethylascorbic acid was prepared by the action of excess diazomethane on ascorbic acid (7); the product was not obtained in crystalline form, but iodine titration showed that only 0.2% reducing material was present.

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[2] We are indebted to Merck & Co., Rahway, N. J., and to Hoffman-La Roche Inc., Nutley, N. J., for a supply of *l*-ascorbic acid, and to Eastman Kodak Company, Rochester, N. Y., for a supply of *l*-ascorbic acid and of *d*-iso-ascorbic acid.

The dehydroascorbic acid was first prepared by titration of ascorbic acid with iodine solution and later by the action of quinone on ascorbic acid (8). Both methods yielded material unsuited to our experiments for reasons which will be discussed later. Satisfactory results were obtained by oxidation of *l*-ascorbic acid (or *d*-iso-ascorbic acid) with Norit in the presence of acetic acid. One hundred milligrams of ascorbic acid was dissolved in 30 cc. of 0.6% acetic acid solution, Norit was added and oxygen was bubbled through the mixture. After 1½ hours the ascorbic acid concentration, judging from the reducing power of the solution, had decreased from 330 mgm % to less than 1 mgm. %. After removing the Norit by filtration sodium bicarbonate was gradually added to the solution until pH 6 was reached This approximately isotonic sodium acetate solution containing the dehydroascorbic acid was used for the experiments.

The *l*-ascorbic acid and *d*-iso-ascorbic acid were estimated in some experiments by iodimetry, in others colorimetrically with 2,6-dichlorophenol indophenol, according to Mindlin and Butler (9), using a Klett-Summerson photoelectric colorimeter.

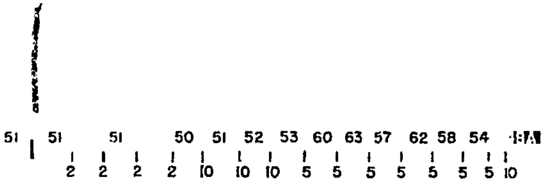


FIG. 2. FROG HEART (CANNULA OF FIG. 1)

[illegible] second intervals.

RESULTS. I. *Effect of l-ascorbic acid.* Altogether 36 experiments were carried out, 24 of these with a concentration of 1:10,000. The characteristic sequence of changes is shown in figure 2.

When *l*-ascorbic acid in an initial concentration of 1:2,000 to 1:100,000 was perfused through the isolated frog heart, the first characteristic change was an increase in the height of contraction. This as a rule occurred without a consistent change in rate. After some time the systolic height of contraction began to decrease, and shortly afterwards, or sometimes simultaneously, the diastolic relaxation became incomplete. The amplitude of contraction was usually further reduced at this time by an increase in rate. In some hearts the acceleration was slight, but in others increases up to 250% of the initial rate were observed. Intensity of systolic effect and increase in heart rate did not run parallel. Finally the ventricle stopped in systole while the atria and the sinus continued beating.

Contrary to our experience with the angelicalactones, it was not possible to establish a clear relation between concentration of ascorbic acid and time taken to cause systolic standstill. It was also noted that dissolving *l*-ascorbic acid in Clark solution made the substance less potent than dissolving it in the bicarbonate buffer solution saturated with the oxygen-carbon dioxide mixture.

The preliminary period of observation during which the heart was supplied with the normal bicarbonate buffer solution usually extended from 10 to 30 minutes. When the continuous replacement of the normal solution was carried on for longer periods up to several hours, the systolic effect and the increase in heart rate still appeared, while a prolonged period of washing decreased or abolished the ability of the heart to respond to the *l*-ascorbic acid solution with an increase in the height of contraction.

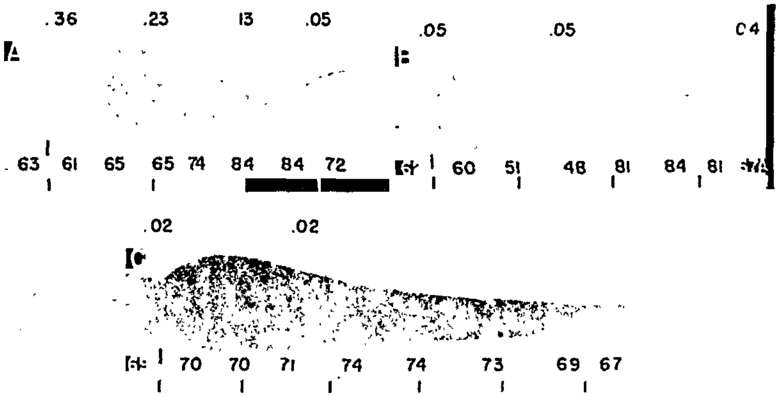


FIG. 3. ISOLATED FROG HEARTS (CANNULA OF FIG. 1)

Bicarbonate buffer solution saturated with 95% oxygen and 5% carbon dioxide. Same *l*-ascorbic acid solution 1:10,000 was used for replacement in hearts A, B, and C. The solution was prepared at 9:55 a.m. At *A*, replacement started 10:23 a.m.; at *B*, replacement started at 12:45 p.m.; at *C*, replacement started at 3:40 p.m. Figures above the tracings represent concentration of *l*-ascorbic acid in mM/liter. Figures below the tracings represent heart rate per minute. Time in half-hour intervals. Room temperature, 24.2–26.7°C.

In a number of experiments a solution with an initial concentration of *l*-ascorbic acid 1:10,000 was run through several hearts successively to obtain information concerning the speed of destruction of *l*-ascorbic acid and the relation between actual *l*-ascorbic acid concentration and intensity of effect. This procedure is illustrated in figure 3. Initially the solution used in this experiment contained 0.55 millimols of *l*-ascorbic acid per liter; when perfusion of heart A was started the concentration was 0.36 millimols per liter. It had reached 0.05 millimols per liter at the time of systolic standstill of heart A. This solution still caused all the characteristic effects in heart B, while in heart C, when the concentration had dropped to 0.02 millimols per liter, or less than 1:200,000, only the initial increase in the amplitude of contraction was observed. While in hearts A and B the heart rate increased, no significant change in rate occurred in heart C.

When the oxidative destruction of *l*-ascorbic acid was prevented by saturating the solution in the Mariotte bottle with the nitrogen-carbon dioxide mixture (bubbling oxygen-carbon dioxide mixture only through the solution in the cannula), the action upon the heart was greatly diminished or entirely abolished. In such experiments the *l*-ascorbic acid concentration, which initially was between 0.5 and 0.55 millimols per liter, decreased not more than 20% within 6 hours, or between 3 and 4% per hour. While the height of contraction diminished gradually, the heart still dilated fully in diastole. When the nitrogen in the Mariotte bottle was replaced by oxygen and destruction proceeded at a high rate, the systolic effect became noticeable after the characteristic latency period; the heart rate increased, and systolic standstill eventually terminated the activity of the heart. Table 1 shows the results of such an experiment. In this case *l*-ascorbic acid content was estimated in the Mariotte bottle as well as in the fluid from the cannula after it had passed the heart. With a replacement rate of 2.1 to 2.5 cc. as in this experiment, the difference in concentration was within the error of the method of estimation, and at this rate of replacement the concentration in the reservoir corresponded closely to the concentration entering and leaving the heart.

TABLE 1

Exp. March 11, 1942. Solution saturated at start with 95 per cent nitrogen and 5 per cent carbon dioxide

TIME	*l*-ASCORBIC ACID CONCENTRATION		HEART RATE	REMARKS
	From bottle	From heart		
min.	*mM/liter*	*mM/liter*		
0	0.5		60	
2				Replacement started
16		0.47	53	Height of excursion diminished
42		0.46	50	Height of excursion diminished
45	0.48			
64	0.40		50	Height of excursion further diminished
66		0.45	50	
70	Oxygenation started in Mariotte bottle with oxygen-carbon dioxide mixture			
73–90			48	Slight increase in height of excursion
109	0 34		58	Height of excursion diminished
110		0.33		
139	0.22		60	
141		0.23		
165			50	Systolic effect begins
181	0.14		45A 14V	Systolic effect marked (A = atria; V = ventricle)
185		0.16	54A	Ventricle stops in systole

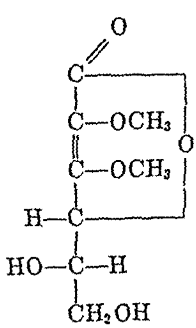
It could be argued that the effect upon the heart might not appear because of a decrease in the oxygen tension of the perfusion fluid, and consequently in the heart itself, although our technique makes this unlikely. That this argument is

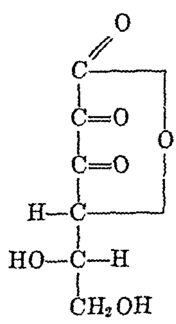
not valid was proved in the following way: A solution of *l*-ascorbic acid 300 milligram % (or about 1:330) was made with bicarbonate buffer solution saturated with 95% nitrogen and 5% carbon dioxide, of which it was found that the reducing power did not change within three hours. This solution was infused continuously through a capillary tubing at a rate of about 0.1 cc. per minute into the wide part of the cannula (at A) through which flowed, at a rate of 2 to 2.5 cc. per minute, bicarbonate buffer solution saturated with 95% oxygen and 5% carbon dioxide. The continuous infusion of the small amount of the concentrated *l*-ascorbic acid solution—constituting about 4% of the total solution—was directed into the vigorous stream of oxygen-carbon dioxide bubbles passing through the fluid in the cannula to ensure uniform distribution. The fluid collected from the side tube (at C) in four such experiments, lasting for 2½ to 3 hours, maintained concentrations of *l*-ascorbic acid ranging from 1:7,000 to 1:9,000 for the duration of the experiments. The only significant change in the activity of the heart observed was a decrease in rate and a diminution of the height of contraction, such as commonly occurred when the experiments were conducted for the same period of time using the bicarbonate buffer solution alone.

II. *Action of d-iso-ascorbic acid.* *d*-Iso-ascorbic acid, or *d*-arabo-ascorbic acid, (II) differs biologically from *l*-ascorbic acid (I), the former having only about one-twentieth of the antiscorbutic activity of the latter (10). While the unsaturated lactone ring is of the same configuration as in (I) the position of the hydroxyl group on C_5 is changed.

In 15 experiments it was found that *d*-iso-ascorbic acid had qualitatively and quantitatively an effect on the frog heart similar to that of *l*-ascorbic acid. The action was diminished or prevented when the oxidative destruction was interfered with by saturating the solution of *d*-iso-ascorbic acid with nitrogen-carbon dioxide mixture.

III. *Action of dimethylascorbic acid and dehydroascorbic acid.* In order to find out whether chemical changes within the lactone ring might be involved in making *l*-ascorbic acid (or *d*-iso-ascorbic acid) active upon the frog heart, dimethylascorbic acid (IV) and dehydroascorbic acid (V) were tested.


(IV)


(V)

Two experiments were made with dimethylascorbic acid in bicarbonate buffer solution saturated with oxygen-carbon dioxide mixture, one with a concentration 1:5,000 (replaced for 4 hours) and one with a concentration 1:3,000 (replaced for 7 hours). No significant effect could be observed on amplitude or rate; and no systolic effect was noticeable at the end of the experiments.

Dehydroascorbic acid prepared by titration of ascorbic acid with iodine solution in acetate buffer was unsuitable for our experiments. Preliminary tests showed that the iodine solution was not toxic to the heart when neutralized and added to the bicarbonate buffer solution in such an amount as would be required to make a solution of dehydroascorbic acid 1:10,000. But the solution of the end-products from *l*-ascorbic acid oxidation with this procedure impaired the activity of the heart, leading to a decrease in the amplitude of contraction and to a marked, although transient, irregularity of the heart rate.

The solutions resulting from the oxidation of *l*-ascorbic acid with quinone proved not toxic; however, they produced results on the frog heart qualitatively similar to those obtained with *l*-ascorbic acid itself under conditions which excluded the presence of *l*-ascorbic acid as well as of dehydroascorbic acid. As these solutions still contained 2 to 2.5% reducing material, it was necessary to ascertain whether or not their slight activity could be attributed to contamination with hydroquinone, particularly as Richter (11) had already shown that quinone and other oxidizing agents exerted a systolic effect upon the isolated frog heart. It was found in six experiments that the action of hydroquinone was similar to that of *l*-ascorbic acid and that concentrations as low as 1:600,000 to 1:1 million caused an initial increase in the height of contraction and that systolic standstill terminated the heart activity within 3 to 6 hours. The effect of hydroquinone, however, was not materially influenced by saturating the solution in the Mariotte bottle with nitrogen-carbon dioxide mixture instead of with oxygen-carbon dioxide mixture.

The preparation of dehydroascorbic acid with Norit in the presence of acetic acid, described above under *methods*, yielded a slightly acid solution devoid of poisonous effects which we believe was sufficiently stable for our purposes. As the halflife of dehydroascorbic acid at 38°C. and pH 7.2 is only two minutes (12) and is probably not very much longer at room temperature (22 to 25°C.) and pH 7.5 to 7.8, it was necessary to apply dehydroascorbic acid to the heart so that it was exposed to the alkaline medium of the bicarbonate buffer solution for only a very short time. This was achieved in the following way: The approximately isotonic sodium acetate solution of pH 6 containing 1:300 dehydroascorbic acid was administered by continuous infusion into part A of the cannula through a capillary tube. The rate of the infusion was between 0.08 and 0.1 cc. per minute. By directing the dehydroascorbic acid solution into the vigorous stream of oxygen-carbon dioxide mixture bubbling through the fluid in A, it was uniformly distributed in the latter in close proximity to the heart. As the bicarbonate buffer solution in the cannula was replaced at the rate of 2 to 2.5 cc. per minute, the diluted dehydroascorbic acid solution was not exposed to the destructive effects of the alkaline medium for more than a very short time.

In this way we feel certain that in 4 different experiments the heart for from 2 to 6 hours actually was under the influence of a concentration of dehydroascorbic acid not too far below the calculated values of 1:7,000 to 1:9,000 and higher than any concentrations of dehydroascorbic acid to which the heart could have been exposed under the conditions of the experiments with *l*-ascorbic acid solutions 1:10,000 described above. Under the influence of the dehydroascorbic acid solution the progressive decrease in the height of systolic contraction was more marked than in control experiments, in which bicarbonate buffer solution alone was used, or in which the sodium acetate solution of pH 6 without dehydroascorbic acid was infused. (Further control experiments showed that the continuous infusion of the sodium acetate solution did not interfere with the action of the ascorbic acid solutions.) Apart from the slight negative inotropic effect dehydroascorbic acid caused no significant change. The same result was obtained with the oxidation product of *d*-iso-ascorbic acid. Dehydroascorbic acid, therefore, cannot be responsible for the action of ascorbic acid on the frog heart.

IV. *Hydrogen peroxide, the cause of the heart action of ascorbic acid solutions.* As neither the two ascorbic acids nor their oxidation products in themselves caused the action upon the heart, and as the blocking of the hydroxyl groups within the lactone ring yielded an inert compound, it seemed possible that the heart effect might be due to a side reaction taking place in the solution during the dehydrogenation of ascorbic acid. This thought was supported by Peugnet's finding that copper was essential for the heart action observed by him.

Guzman Barron, DeMeio, and Klemperer studied the catalytic action of copper (Cu^{++}) upon the dehydrogenation of ascorbic acid (13) and, on the basis of observations by Traube (14) and by Wieland and Franke (15), postulated the appearance of hydrogen peroxide in the solution during the reoxidation of the reduced catalyst (Cu^{+}). The presence of hydrogen peroxide in ascorbic acid solutions was actually demonstrated by Holtz and Triem (16) and by Schales (17). As was already mentioned, oxidizing agents among them certain peroxides, were found to have a systolic action upon the isolated heart of the frog (11).

1. If hydrogen peroxide was responsible for the heart action of solutions of *l*-ascorbic acid or *d*-iso-ascorbic acid, then a destruction of hydrogen peroxide immediately upon its formation should prevent the effect. When two drops of frog blood (2 experiments) or 0.05 cc. of a solution of a highly purified catalase preparation (4 experiments) was added to 500 cc. of a 1:10,000 solution of *l*-ascorbic acid, although the chemical analyses indicated that dehydrogenation took place, the characteristic action upon the heart was prevented.

As Holleman first observed, pyruvic acid and hydrogen peroxide react very rapidly to form acetic acid, carbon dioxide, and water (18–20). Lipmann investigated the speed of the reaction at various concentrations of pyruvic acid. At a concentration of 0.1% the rapidity of the reaction was of the same order as that of the destruction of hydrogen peroxide by catalase. Sevag (22) utilized pyruvic acid to protect the enzymatic activity of pneumococci against the destructive effect of hydrogen peroxide, which is formed during the culture of many

microörganisms (23). If to an ascorbic acid solution 1:10,000, sodium pyruvate was added in a concentration of 0.1%, the characteristic heart action did not occur, while the decrease in the reducing power of the ascorbic acid solution was not prevented.

2. The appearance of hydrogen peroxide in *l*-ascorbic acid solution has been ascertained qualitatively (16, 17, 24). We attempted an approximate quantitative estimation employing for comparison hydrogen peroxide solutions of known strength in distilled water and using the luminol reaction of Lommel (25). This method is based on the appearance of luminescence when hydrogen peroxide reacts with *3*-aminophthal-hydrazide (luminol). The reagent as employed by Schales (24) contained, in 100 cc.: luminol 0.1 gram; mesohemin 0.003 gram; and sodium carbonate 1 gram. Five cubic centimeters were added to 10 cc. of *l*-ascorbic acid solution or to 10 cc. of an appropriate dilution to make it possible to see the luminescence in the dark-room at a distance of 50 cm. from the eye of the observer.

Three experiments with *l*-ascorbic acid and one with *d*-iso-ascorbic acid were made by the usual method but without a frog heart attached to the cannula. Five to ten minutes after preparing the ascorbic acid solution 1:10,000 in bicarbonate buffer solution saturated with oxygen-carbon dioxide mixture, hydrogen peroxide was found in the solution in the Mariotte bottle in a concentration of about 1:10 million. Administration was then started to the cannula where a stream of oxygen-carbon dioxide mixture bubbled through the solution. Within 20 minutes the hydrogen peroxide concentration in the solution in the cannula approached 1:500,000, and 10 minutes later it was found to be 1:250,000. One hour after the beginning of the experiment a concentration of between 1:200,000 and 1:100,000 was reached, and this was maintained for the second hour. After that the concentration began to decrease.

3. The range of concentration of hydrogen peroxide causing the characteristic effect upon the frog heart was established under our experimental conditions in ten experiments. As hydrogen peroxide is not stable in an alkaline medium we administered it in the way described for dehydroascorbic acid, dissolving it in an 0.8% sodium acetate solution of pH 6 and infusing the solution continuously into part A of the cannula at a rate of 0.08 to 0.1 cc. per minute. The actual concentrations reaching the heart were between 1:100,000 and 1:1 million. As in the experiments with ascorbic acid the initial increase in the height of contraction and the final systolic standstill could be observed. An increase in heart rate occurred in some experiments only. While a concentration of 1:500,000 caused systolic standstill within a period of about 3 hours, a range of concentration of hydrogen peroxide between 1:100,000 to 1:300,000 led to systolic standstill within 1 to 2 hours as was observed with ascorbic acid solution of an initial concentration of 1:10,000.

The results of these three series of experiments are consistent with the assumption that the characteristic heart action of ascorbic acid solutions is caused by hydrogen peroxide.

SUMMARY

l-Ascorbic acid or *d*-iso-ascorbic acid, when administered continuously to the isolated frog heart in an initial concentration of 1:10,000, led to an increase in the height of contraction and an inconsistent increase in rate, and caused irreversible systolic standstill within 1 to 2 hours. Our experiments indicate that the effect upon the frog heart was not due to *l*-ascorbic acid or *d*-iso-ascorbic acid itself, nor to their products of dehydrogenation, the corresponding dehydroascorbic acids. The effect was absent when the ascorbic acids were protected against the dehydrogenation by excluding oxygen from the solution, or, in the case of *l*-ascorbic acid, by introducing methyl groups into the hydroxyl groups of the lactone ring to form the dimethyl ascorbic acid. During the process of dehydrogenation of *l*-ascorbic acid or *d*-iso-ascorbic acid 1:10,000 in bicarbonate buffer solution, hydrogen peroxide appeared in the solutions and was found to reach concentrations of the range of 1:200,000 to 1:100,000 under the conditions of our experiments. Evidence is presented which indicates that the characteristic action of *l*-ascorbic acid and of *d*-iso-ascorbic acid solutions upon the frog heart can be accounted for by these concentrations of hydrogen peroxide.

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THE EFFECT OF COCAINE, ERGOTAMINE AND YOHIMBINE ON THE ACTIVITY OF PHENOL SULFUR ESTERASE

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It is generally assumed that the inactivation of epinephrine *in vivo* is an enzymatic process. The enzyme involved is not yet identified, but it is believed to be an oxidase. The review of literature is omitted because of the numerous recent reviews of the subject. That the inactivation of epinephrine is due only to oxidative processes has been questioned recently mainly on the following grounds:

1) The distribution of oxidative enzymes in various tissues does not parallel the ability of the tissues to destroy epinephrine (1–3). The rate of oxidation by the enzymes is probably too slow (4).

2) The degree to which a substance potentiates epinephrine often does not coincide with the degree to which it inhibits oxidative processes. Conversely the degree to which a substance decreases the effect of epinephrine often does not coincide with the degree of increase of the oxidative processes. Affinity to oxidative enzymes and biological effect often differs significantly with some of the epinephrine-like substances (5).

3) Clark and Raventos (6) calculated that only 50% of the inactivation of epinephrine *in vivo* could be attributed to the action of oxidative enzymes.

In 1940 Richter (3, 7) found that epinephrine administered by mouth can be recovered from the urine as a sulfur ester. This indicates that a sulfatase plays a part in the inactivation of epinephrine. Among the known enzymes, phenol sulfur esterase is probably the one to which this process could be attributed.

This paper concerns itself with the effect of cocaine, ergotamine and yohimbine on the activity of the phenol sulfur esterase to determine whether or not this effect parallels their influence on the effect of epinephrine *in vivo*. Our work does not give a final answer to the question of whether or not phenol sulfur esterase is involved in the inactivation of epinephrine, but suggests that it may be.

METHODS. The enzyme activity of phenol sulfur esterase was investigated *in vitro*.

The enzyme was obtained from fresh cat muscle prepared according to the method described by Neuberg (see 8). 1.25% potassium phenol sulfonate was used as substrate. This salt was prepared according to the method of Verley and Czepek (see 8).

Two cc of muscle extract, 8 cc. of the substrate, 500 mgm. of calcium carbonate and 1 drop of toluol were incubated for 3 days at 30°C. To some samples of this mixture cocaine, ergotamine tartrate or yohimbine were added before incubation to make a concentration of 5 per cent. The activity of the phenol sulfur esterase was measured by the amount of phenol liberated. Because phenol is very soluble in toluol, the toluol content of the samples was kept at a minimum. Large amounts of calcium carbonate were added, first to speed

up the decomposition process by neutralizing the other end product, sulfuric acid, and secondly, to keep the pH of the solution as constant as possible (9, 10). The activity of the phenol sulfur esterase and the hydrolysis of the substrate vary with a change of the pH. To avoid error from this source the pH of the samples was checked every hour during the incubation period and corrected when necessary. This precaution was important for another reason: in our experiments the presence of large amounts of cocaine, ergotamine or yohimbine, and their decomposition products, might cause a change of the pH which alone would modify the enzyme activity.

For controls two series of samples containing the above substances were used, one without enzyme and one without substrate.

The free phenol content of samples taken before and after incubation was determined by the method of Theis and Benedict (see 11). Both tungstate and zinc hydroxide solutions were used to precipitate the proteins. The pH of the solutions must be corrected carefully to the same level before the addition of the para-nitroaniline dye because the intensity of the color developed depends not only on the amount of phenol present, but also on the pH. A photoelectric colorimeter was used to determine the phenol content of the solution. The phenol values given in the table represent the difference between the phenol content of the control solutions and the phenol content of the experimental samples. They are given in mgm. per hundred cc. of solution.

TABLE 1

Effect of cocaine, ergotamine and yohimbine on the activity of phenol sulfur esterase

ENZYME SUBSTRATE	NO. OF EXPERIMENTS	PHENOL LIBERATED mgm./100 cc.		ACTIVITY IN % OF CONTROL
		Mean	S.E.*	
Control	67	9.6	±0.30	100
Cocaine	54	5.3	±0.40	55
Ergotamine	10	12.8	±0.69	128
Yohimbine	10	12.0	±0.69	125

* S.E. of mean $= \sqrt{\dfrac{\Sigma(\Delta)^2}{N(N-1)}}$

Σ = summation.

Δ = individual deviation from mean.

N = number of experiments.

The amount of substrate used is sufficient to liberate about a hundred times as much phenol as was liberated in the given experiments.

RESULTS. The amount of the phenol liberated in the decomposition of potassium phenol sulfonate served as indicator of the activity of phenol sulfur esterase. The data are summarized in table 1.

During a 3-day incubation period the enzyme liberated about 1.5–2.5% of the phenol content of the substrate. The enzyme activity was decreased about 44.8% in the samples containing cocaine, was increased about 28% in the samples containing ergotamine and was increased about 25% in the samples containing yohimbine. A statistical treatment of the data indicates that these differences are significant and not due to chance.

DISCUSSION. The activity of phenol sulfur esterase has been investigated in

the absence and in the presence of cocaine, ergotamine and yohimbine. Cocaine decreases the activity of the above enzyme, while ergotamine and yohimbine increase it.

These results support, in an indirect way, Richter's theory that in the inactivation of epinephrine a sulfur esterase may be involved. The phenol sulfur esterase content of the epinephrine sensitive organs has been only partly investigated (12–14), but up to the present time they do not disprove the possibility of a distribution related to the epinephrine sensitivity of the tissue.

The results of many previous investigations make it likely that in the liver, and in some other tissues, epinephrine is inactivated, mainly by oxidases, but experiments with the liver excluded (15, 16) indicate that the rest of the body also plays an important part in the inactivation of epinephrine. It is possible that organs poor in oxidases but sensitive to epinephrine dispose of the drug through esterification with sulfuric acid. This hypothesis appears not improbable when we consider the mechanism of disposal of other phenols in the body (17–30), and the potentiation of the effect of epinephrine by the phenols (31–34). Phenols may compete for the same enzyme, possibly for the sulfur esterase.

SUMMARY

1. The effect of cocaine, ergotamine and yohimbine on the activity of the phenol sulfur esterase has been investigated.

2. Cocaine inhibits the activity of this enzyme while ergotamine and yohimbine increase it.

3. This study suggests that the inactivation of the phenol sulfur esterase is one mechanism by which cocaine potentiates the action of epinephrine, and a potentiation of the activity of phenol sulfur esterase is one mechanism by which ergotamine or yohimbine depress the action of epinephrine.

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SULPHAMETHAZINE (2-4′-AMINOBENZENESULPHONYLAMINO-4:6-DIMETHYLPYRIMIDINE)

A NEW HETEROCYCLIC DERIVATIVE OF SULPHANILAMIDE

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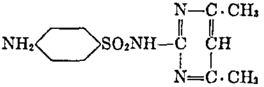
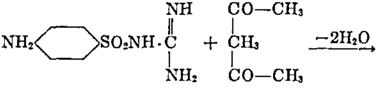
The introduction of heterocyclic groups into the sulphanilamide molecule has resulted in substances of more potent antibacterial activity and wider range, the most important of the earlier derivatives being sulphapyridine and sulphathiazole. This increase in activity was, however, associated with a higher incidence of toxic symptoms, for example, nausea, vomiting and renal disturbance, the last-named being related to the low solubility of the acetylated drugs (1) (2). More recently the sulphanilamide derivative of 2-aminopyrimidine has been introduced in America under the name sulphadiazine (3), which causes far less nausea and vomiting than the other drugs but which is not free from the risk of renal complication (4). During the course of our researches we prepared a near relation of sulphadiazine, namely, 2-4′-aminobenzenesulphonylamino-4:6-dimethylpyrimidine to which we gave the name *sulphamethazine.*



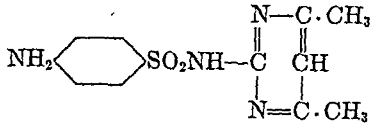
Sulphamethazine

The purpose of this paper is to record the experimental comparisons of this substance with sulphanilamide and sulphapyridine carried out prior to and during its clinical trial, the results of which have been reported elsewhere (5).

Chemical and physical properties. Sulphamethazine is readily prepared by heating together at 130°C. equimolecular amounts of sulphanilylguanidine and acetylacetone. It is precipitated in the presence of water in the form of very pale yellow crystals of the hemihydrate.



Sulphanilylguanidine Acetylacetone



Sulphamethazine

Since we began the present studies with this substance its preparation by a different route has been recorded by Caldwell *et al.* (6) and Roblin *et al.* (7). The former gives a melting range of 178–180°C. (corr.) and the latter 198°–199°C. (corr.). Our first preparations conformed to the lower figures, but later samples prepared by the same process melted at 197–198°C. (corr.), and since then we have been unable to reproduce material melting at the lower temperature. We are unable as yet to offer any explanation of this phenomenon beyond the empirical suggestion that the low melting form of the solid is metastable.

Sulphamethazine has both basic and acidic properties. Solutions of the sodium salt of sulphamethazine at a concentration of 1 gram in 3 cc. (pH 9.7) may be sterilized without decomposition. When diluted to 8 cc. with water, this solution has a pH of 9.5 and has been injected intravenously and intramuscularly without causing irritation. (The pH values of 10% solutions of the sodium salts of sulphapyridine and sulphadiazine are 10.7 and 10.2 respectively (8).)

In vitro EXPERIMENTS. It is well known that the extent of the *in vitro* action of sulphonamides is greatly dependent upon the conditions in the following eriments, but the results are, of course, comparative only.

ıspensions of the test substances were prepared by thorough grinding of 50 mgm. with a ll amount of sterile distilled water, followed by dilution to a volume of 5 cc., giving a 0 suspension. Serial dilutions with a ratio of 1:3 were prepared from this in distilled ɪr. One-tenth cc. amounts of each dilution were placed in 3″ x ½″ test tubes. When all lilutions of each substance had been dispensed in this way, a very dilute suspension of ;est organism was prepared in sufficient medium to permit 1 cc. to be transferred to each ıe small tubes. For experiments with *Streptococcus pyogenes*, Kruger strain (Group A), with *Streptococcus pneumoniae* Type 1, the medium was Wright's broth (9) prepared veal or beef enriched with 5% of defibrinated horse-blood. The experiments with *erium friedländeri* were carried out in Wright's broth without modification. In preparthe diluted suspensions of test organisms, 24-hour cultures in the test medium were used, diluted to give 200–500 colonies when 1 cc. was plated in plain or blood-agar. The stock cultures were maintained in the same media with weekly sub-cultures. This method of working ensured that each tube contained the same number of viable organisms. After thorough mixing, all tubes were incubated at 40°C. (10) for the streptococcal and pneumococcal experiments and at 37°C. for Friedländer's bacillus. After 24 hours the tubes were examined for turbidity or haemolysis before being shaken. Then each tube was shaken and one loopful from each was streaked on the same media as used in the test, solidified with 2% agar. In the following tables (1, 2 and 3) only the results of this plating are shown. The results are typical of many others. (The following degrees of growth were recognized: trace ∓, ±, +.)

The above experiments indicate that the activities of both sulphapyridine and sulphamethazine towards streptococci and pneumococci are approximately

equal, both being rather more active than sulphanilamide towards streptococci. This is a result we have frequently obtained, although on other occasions sulphanilamide has appeared relatively less active. Inhibition extending over a wide range of concentrations as shown in tables 1 and 2 has also been observed very frequently. Table 3 records the effect of three compounds upon Bact. friedländeri under similar conditions. Sulphamethazine appears definitely more active than sulphapyridine and very much more active than sulphanilamide against this organism.

TABLE 1

Streptococcus pyogenes

TEST SUBSTANCE	CONCENTRATION—ONE PART IN						CONTROL
	1,000	3,000	9,000	27,000	81,000	243,000	
Sulphanilamide	∓	∓	∓	±	+	+	+
Sulphapyridine . .	−	−	−	±	+	+	
Sulphamethazine	−	−	−	trace	+	+	

TABLE 2

Streptococcus pneumoniae

TEST SUBSTANCE	CONCENTRATION—ONE PART IN						CONTROL
	1,000	3,000	9,000	27,000	81,000	243,000	
Sulphanilamide...... .. .	∓	∓	±	±	+	+	+
Sulphapyridine . .	∓	∓	±	±	±	+	
Sulphamethazine	∓	∓	±	±	±	±	

TABLE 3

Bact. friedländeri

TEST SUBSTANCE	CONCENTRATION—ONE PART IN						CONTROL
	1,000	3,000	9,000	27,000	81,000	243,000	
Sulphanilamide .	−	∓	+	+	+	+	+
Sulphapyridine . .	−	−	−	+	+	+	
Sulphamethazine .	−	−	−	−	+	+	

In vivo EXPERIMENTS. The general technique for carrying out these experiments was the same for both streptococcal and pneumococcal infections.

Groups of 12 mice weighing between 18 and 25 grams were used. All doses of drugs were given in 1 cc. water, the sulphanilamide in solution (the 10 mgm. per cc. dose being kept at 37°C.) and the other substances in suspension. Sulphapyridine and sulphamethazine were prepared as fine dispersions by ballmilling overnight, using a non-toxic dispersing agent All doses were administered by mouth using a 2 cc. syringe attached to a short length of ureteral catheter by means of a cut-off needle. The distal end of the catheter was rounded and coated with rubber solution. The first dose of drug was given 1 hour

before infection, the second 5 hours later and then three more doses were given 24, 48 and 72 hours after infection.

The same strains of streptococcus and of pneumococcus as were used in the experiments already described were employed but had been maintained continuously by mouse-to-mouse passage every other day. Freshly isolated 18-hour cultures in Wright's broth

TABLE 4

Infection with Streptococcus pyogenes, Kruger strain

DRUG	DOSE	DEATHS ON DAYS FOLLOWING INFECTION								TOTAL DEATHS	AVERAGE SURVIVAL TIME
		0	1	2	3	4	5	6	7		
	mgm./20 grams										*days*
Sulphanilamide	2		5	7						12	0.9
	5			6	6					12	1.5
	10			2	6	1		3		12	2.7
Sulphapyridine	2		1	9	2					12	1.2
	5			1	4	3	1	3		12	3.1
	10				1	1	5	3	1	11	4.4
Sulphamethazine	2			11	1					12	1.1
	5				6	3		2	1	12	3.1
	10				1		2	8	1	12	4.7
Untreated controls			12							12	0.8

TABLE 5

Infection with Streptococcus pneumoniae, Type 1

DRUG	DOSE	DEATHS ON DAYS FOLLOWING INFECTION								TOTAL DEATHS	AVERAGE SURVIVAL TIME
		0	1	2	3	4	5	6	7		
	mgm./20 grams										*days*
Sulphanilamide	2			10	1					11	1.6
	5			3	5	2	1			11	2.5
	10				1	1	6	1		9	4.6
Sulphapyridine	2			7	5					12	1.4
	5			1	7		2			10	3.1
	10						4	2	1	7	5.6
Sulphamethazine	2			1	9		1			11	2.5
	5			1	2	1	5	2		11	3.8
	10						4	3	1	8	5.4
Untreated controls				9	3					12	0.8

containing 5% horse-blood provided the infection. For use these cultures were diluted in plain broth and injected in a dose of 0.25 cc. intraperitoneally. The streptococcal culture was diluted 1/100 and the pneumococcal culture 1/10,000. All mice were housed individually and kept in a constant-temperature room. Deaths were recorded for the first seven days and average survival times calculated according to Whitby (11). The results are shown in tables 4 and 5.

From tables 4 and 5 it will be seen that the therapeutic effects produced by sulphapyridine and sulphamethazine were almost identical. Against the streptococcus both compounds were rather more effective than sulphanilamide at each dose level. Against pneumococci the difference between sulphanilamide on the one hand and sulphapyridine and sulphamethazine on the other is less marked. This has been our general experience.

PHARMACOLOGY. The efficiency of absorption of sulphanilamide drugs and their ability to maintain an effective concentration in the blood are important factors governing therapeutic effect. Information on these points is readily gained by the use of small animals, although the results obtained are not always

FIG. 1. BLOOD CONCENTRATION/TIME CURVES IN MICE FOR SULPHANILAMIDE DRUGS FOLLOWING A SINGLE ORAL DOSE OF 5 MGM./20 GRAMS

exactly paralleled by experience in human subjects. Blood level curves obtained after oral and intravenous administration to mice of the same dosage of sulphanilamide, sulphapyridine and sulphamethazine are given below in figures 1 and 2.

Sulphanilamide was given orally as a 0.5% solution and sulphapyridine and sulphamethazine as 0.5% suspensions in water, at the rate of 5 mgm./20 grams mouse. Intravenous injections at the same dose level were made into a tail vein using sulphanilamide in the form of a 1% solution, and sulphapyridine and sulphamethazine as solutions of the sodium salts containing 1% of the free drug. Groups of three mice were employed and pooled-blood level determinations were made by the method of Rose (12).

The curves obtained after intravenous injection of the drugs illustrate the comparatively slow rate at which sulphamethazine is eliminated from the

blood stream. Sulphapyridine is intermediate between the latter and sulphanilamide. The results are reflected in the oral absorption curves. Sulphamethazine attains a higher maximum than sulphanilamide and the concentration is maintained at a higher level throughout the length of the experiment. Sulphapyridine is less rapidly absorbed than either of the other drugs.

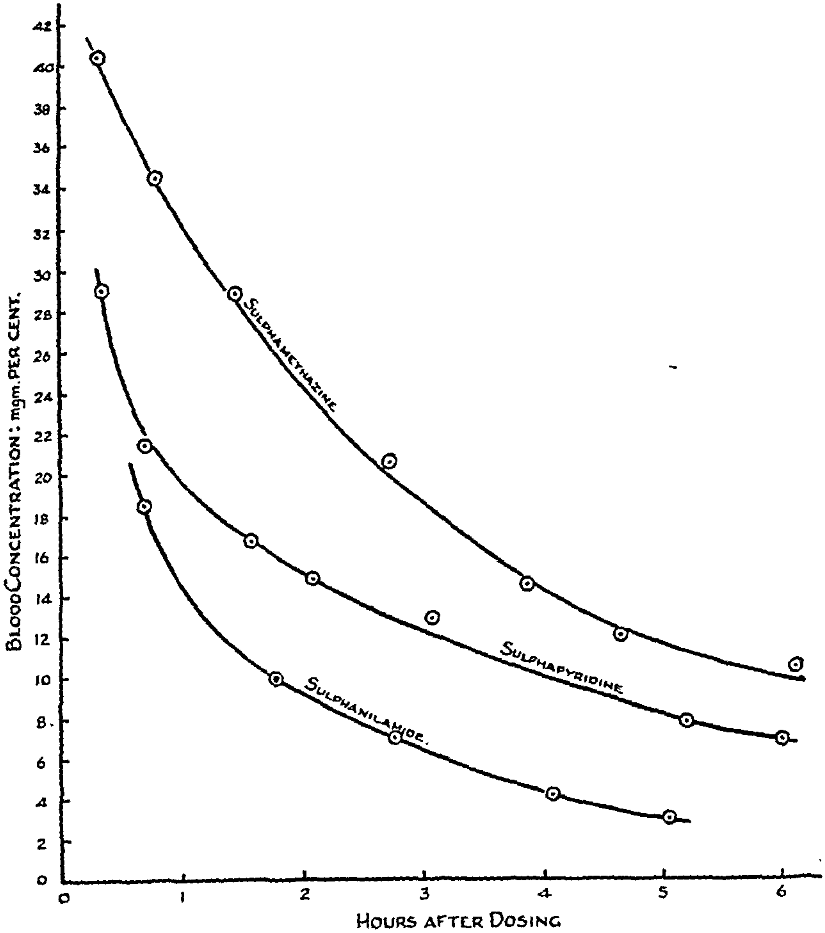


FIG. 2. BLOOD CONCENTRATION/TIME CURVES IN MICE FOLLOWING SINGLE INTRAVENOUS DOSES OF 5 MGM./20 GRAMS OF SULPHANILAMIDE, SULPHAPYRIDINE AND SULPHAMETHAZINE (SODIUM SALTS)

TOXICITY. No attempt has been made to determine median lethal doses accurately since we have found that wide differences in susceptibility exist between different strains of mice. We have been content to compare the effects of similar doses of the three compounds on groups of six male mice of similar weight taken from the same stock.

Probably the best measure of the intrinsic toxicity of the three compounds under discussion is given by the intravenous injection of solutions in sodium hydroxide, as by this means differences due to different rates of absorption are eliminated. Injections were made into a tail vein at a constant volume of 0.2 cc., each injection taking 10–12 seconds. The results are shown in table 6.

The toxicity of all compounds appears substantially the same.

In view of the statement of Marshall and Litchfield (13), that the acetyl derivative of sulphapyridine is more toxic than the parent compound, the acetyl derivatives of the three compounds in which we were interested were

TABLE 6

Deaths following intravenous injections of sodium salts in groups of 6 mice

DOSE	SODIUM SALT OF		
	Sulphanilamide pH 10.0	Sulphapyridine pH 10 0	Sulphamethazine pH 9.5
mgm /20 grams			
10	0	0	0
12	2	1	0
14	4	3	4
15	5	4	5
16	6	6	6

TABLE 7

Deaths following intravenous injection of sodium salts of acetyl derivatives in groups of 6 mice

DOSE	SODIUM SALT OF		
	Acetylsulphanilamide pH 10.0	Acetylsulphapyridine pH 10.0	Acetylsulphamethazine pH 8 5
mgm /20 grams			
14	0		
16	4	0	
17	6		
20		3	0
22		6	
25			1
30			5

compared as in the previous experiments, i.e., solutions of the sodium derivatives were administered intravenously. The results are shown in table 7.

These results do not support Marshall's contention, the toxicity of acetylsulphapyridine being considerably less than that of the parent compound at any rate in mice. They suggest that the toxicities of the acetyl derivatives of sulphapyridine and sulphamethazine are about two-thirds and one-half respectively of the parent compounds.

Finally, the acute oral toxicity of the three compounds given as suspensions was compared. The suspensions were prepared and administered as for the therapeutic experiments described above. These results are shown below in table 8.

The low toxicity of sulphapyridine when given by mouth is due to its poor absorption. On the other hand sulphamethazine is well absorbed, yet is of low toxicity.

Summing up the pharmacological data, we may say that, in mice, sulphamethazine is very rapidly absorbed from the intestinal tract and is more slowly excreted than either sulphanilamide or sulphapyridine. The acetyl derivative of sulphamethazine is less toxic than the acetyl derivatives of the other two compounds.

EXCRETION OF SULPHANILAMIDE DRUGS. (*a*) *Water-solubility.* Sulphanilamide drugs are excreted partly as the free amines and partly as the acetyl derivatives. Both forms of sulphanilamide are sufficiently soluble in water to be concentrated in the urine without precipitation in the renal system. On the other hand, acetylsulphapyridine and acetylsulphathiazole are much less soluble and frequently occur in the urine in solid form. This often results in toxic symptoms such as haematuria or even anuria. Similar renal complications have also resulted from the administration of sulphadiazine.

TABLE 8

Deaths following oral administration of suspensions of parent compounds in groups of 6 mice

DOSE	SULPHANILAMIDE	SULPHAPYRIDINE	SULPHAMETHAZINE
mgm./20 grams			
50	0		0
60	1		0
70	0		0
80	1	0	1
90	6		1
100	5	0	2

Water-solubility curves for several of the well-known sulphanilamide compounds and their acetyl derivatives have been constructed over a range of pH values in order to indicate whether any risk of renal disturbance might be expected to be associated with the administration of sulphamethazine.

The values were obtained by stirring an excess of each drug in boiling twice-distilled water, cooling to 37°C. and thermostatically maintaining that temperature, then adding 0.1 N caustic soda to increase the pH. The pH was measured by means of a glass electrode-calomel half-cell system, and was permitted to reach equilibrium before a reading was taken. The concentration of the drug in solution was determined colourimetrically by withdrawing a sample through a filter-tip into a preheated micro-pipette. In the case of the acetyl derivatives, de-acetylation was necessary before the colourimetric estimation, which depended upon a diazo reaction, could be made. The results obtained with the free amines are shown in figure 3 and with the acetyl derivatives in figure 4.

The higher water-solubility of acetyl sulphadiazine as compared with that of the parent amine recorded by Roblin *et al.* (3), is substantiated by our observations. Moreover, this phenomenon extends over the whole pH range of the experiment. The solubilities of the remaining acetyl compounds are in each

case lower than those of the corresponding amines, but the solubility ratio is not even approximately constant. Thus sulphathiazole is more soluble than either sulphapyridine or sulphadiazine, but acetylsulphathiazole is the least soluble of all the acetylated derivatives. Further, it would appear that the

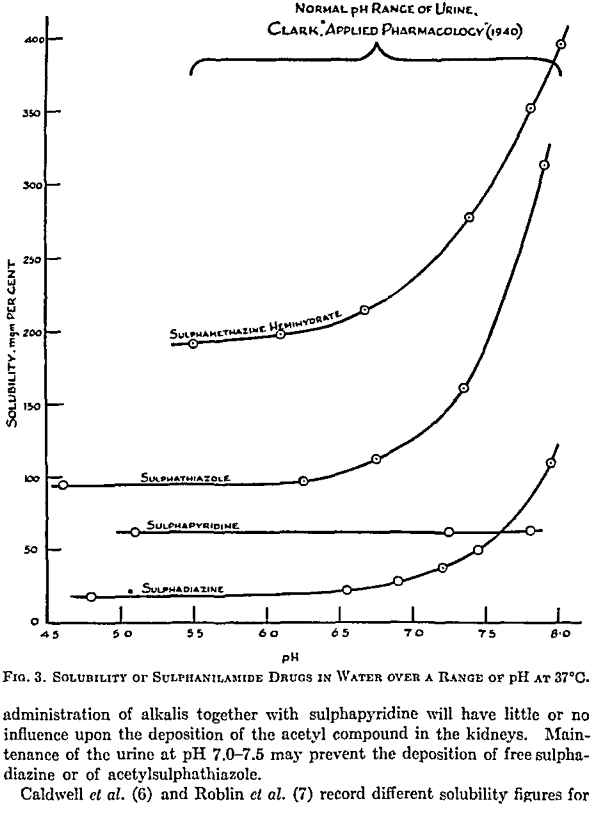


FIG. 3. SOLUBILITY OF SULPHANILAMIDE DRUGS IN WATER OVER A RANGE OF pH AT 37°C.

administration of alkalis together with sulphapyridine will have little or no influence upon the deposition of the acetyl compound in the kidneys. Maintenance of the urine at pH 7.0–7.5 may prevent the deposition of free sulphadiazine or of acetylsulphathiazole.

Caldwell *et al.* (6) and Roblin *et al.* (7) record different solubility figures for

sulphamethazine in water. The former give 150 mgm./100 cc. at 29°C. and the latter 75 mgm./100 cc. at 37°C. but state "compound tends to remain supersaturated indefinitely in the presence of excess solid. Value represents approximate equilibrium when approached from low side." We believe that

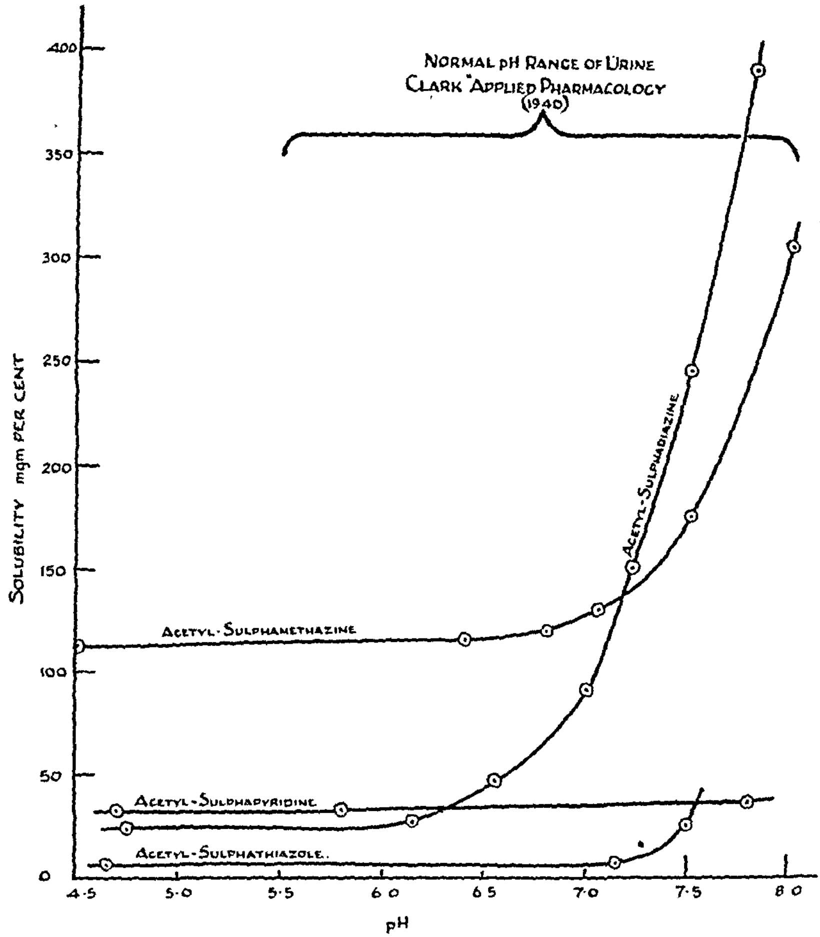


FIG. 4. SOLUBILITY OF CONJUGATED (ACETYLATED) SULPHANILAMIDE DRUGS IN WATER OVER A RANGE OF pH AT 37°C.

the higher figures obtained by Caldwell and his co-workers, and in our own experiments, may be due to the fact that the solid in equilibrium with the solution is the hemihydrate, whereas Roblin employed the anhydrous compound, hydration being only partially complete at the time at which the solubility measurement was made.

Sufficient data are not yet available to predict accurately the minimum solubility of these drugs or their acetyl compounds that is necessary to avoid renal disturbance. Since sulphanilamide is free from this drawback and sulphapyridine is not, the critical region must lie between the solubilities of the acetyl derivatives of the two compounds, namely, 250 mgm./100 cc.(approx.) and 34.1 mgm./100 cc. respectively, at 37°C. and pH 6.5. The corresponding solubility of acetylsulphamethazine is 117 mgm./100 cc., or roughly midway between these figures, and appears to be high enough to prevent deposition of this substance in the urinary system of man following normal dosage levels of the parent drug.

(*b*) *Kidney damage demonstrable in rats.* The effects of repeated, large, oral doses of sulphamethazine on the kidneys were examined. Groups of twelve young rats were given daily doses of 1 gram per kilo of one or other compound. No histological changes were observed in the organs of animals of either group killed after 14 days. The remaining rats were killed on the 29th day (by which time each animal had received approximately 6 grams of drug) and examined.

All the kidneys from the six rats in the sulphamethazine group and the kidneys from four of the six rats in the sulphapyridine group were normal. In two of the rats who had received sulphapyridine there were bilateral, diffuse changes of extreme severity which resembled those produced by the administration of certain heavy metals or oxalates. They are pathologically identical with the changes described in human kidneys following treatment with sulphathiazole (14).

It should be noted that the kidneys of the rats in the sulphamethazine group were subjected to a strain very much more severe than would appear simply from a consideration of the amounts of the two drugs given by mouth. This was apparent from the results of analyses of blood samples carried out at intervals during the period of treatment. In the sulphapyridine group, blood concentrations fluctuated between 15–20 mgm./100 cc. (2 hours after dosing) and 2–4 mgm./100 cc. (23 hours after dosing). The corresponding figures for sulphamethazine were 25–30 mgm./100 cc. and 8–12 mgm./100 cc.

Clinical pharmacology. During the clinical trial of sulphamethazine referred to above (5), quantitative studies of the distribution of the compound in various body fluids were made on both normal and sick patients.

The analyses of sulphamethazine were carried out on 0.2 cc. of capillary blood by the method of Rose (12) using double quantities of reagents. Urinary analysis was by the same method. In the determination of the conjugated compound, the protein-free filtrate was hydrolyzed for 1 hour by hydrochloric acid and the mineral acidity then neutralised by the equivalent quantity of caustic soda.

To determine the fate of the drug in man, five convalescent patients were given varying doses. Three patients received 1 gram of the sodium salt of sulphamethazine intravenously. One is represented in figure 5 inset. The second gave very similar results but the third excreted the drug more slowly, 1.4 mgm. % remaining in the blood after 10½ hours. Between 50 and 62%

of the drug was recovered from the urine in 20 hours. The patient represented in figure 5 excreted 57.6 % of the drug in $17\frac{1}{2}$ hours after 2 grams given intravenously and 46% in 21 hours after 4 grams by mouth. About 50% of these recoveries were in the free form.

Routine estimations of sulphamethazine were made on a series of 102 adult patients receiving 4 grams orally followed by 2 grams six hourly, the treatment generally used by Macartney *et al.* Blood levels were usually examined twice daily and in 36 cases analyses of the urinary output were made. Whenever

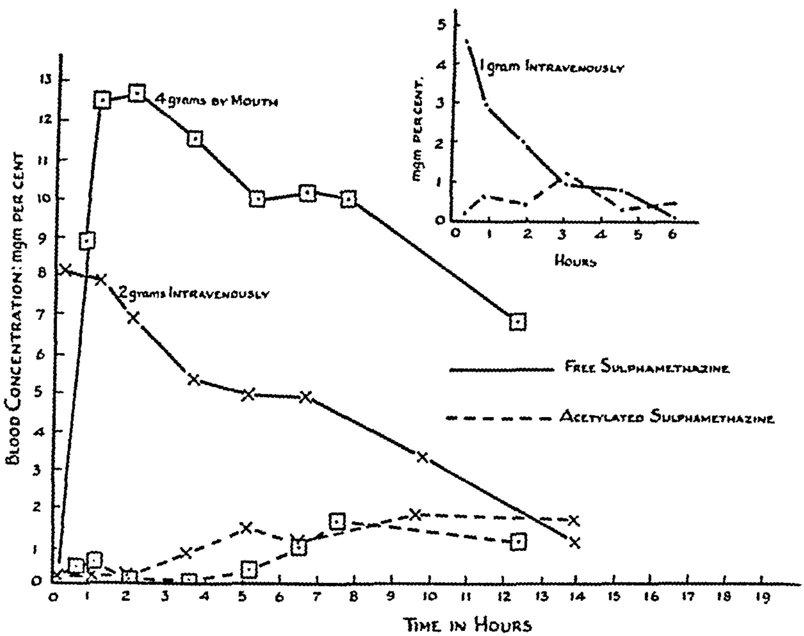


Fig. 5. Blood Concentrations after 2 Grams Sodium Salt Intravenously and after 4 Grams by Mouth in the Same Patient
Inset, blood concentrations after 1 gram sodium salt intravenously

possible cerebrospinal fluids and pleural fluids were examined. Both free and conjugated sulphamethazine were estimated.

Some patients showed consistently high and some consistently low blood levels. During the routine treatment the blood concentration of sulphamethazine in most patients was between 5 and 10 mgm. % (fig. 6). One typical case is shown in detail in figure 7. The level of the conjugated compound was very seldom higher than 5 mgm. %. High concentrations of the conjugated compound usually occurred with low concentrations of free sulphamethazine. Similar blood levels were found in a small series of cases treated with an initial dose of 4 grams followed by 1 gram four hourly.

The urines contained very variable quantities of sulphamethazine (up to 1%) but usually less than half of this was in the free form. The ratio of free to conjugated sulphamethazine was invariably much lower in the urine than in the blood.

A number of patients whose initial condition on admission to hospital was critical or who failed to respond in the usual way to routine dosage by mouth were given a more intensive course of treatment, usually 2 grams every four hours by mouth, together with intravenous or intramuscular injections of the sodium salt. One case of this type, considered to be in extremis, with vomiting,

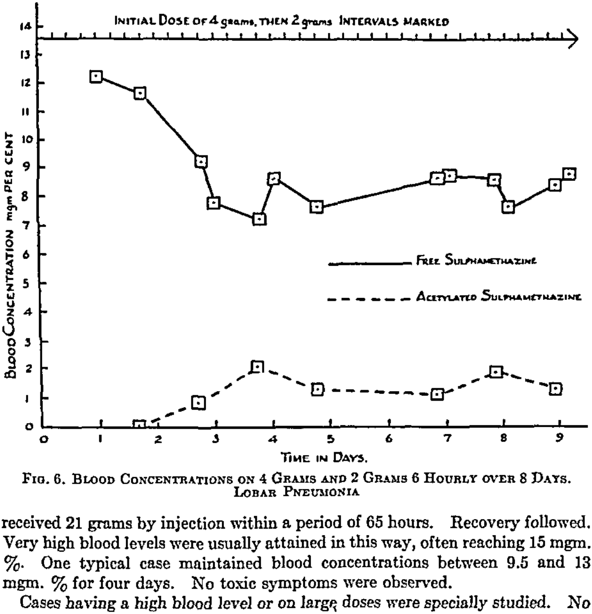


Fig. 6. Blood Concentrations on 4 Grams and 2 Grams 6 Hourly over 8 Days. Lobar Pneumonia

received 21 grams by injection within a period of 65 hours. Recovery followed. Very high blood levels were usually attained in this way, often reaching 15 mgm. %. One typical case maintained blood concentrations between 9.5 and 13 mgm. % for four days. No toxic symptoms were observed.

Cases having a high blood level or on large doses were specially studied. No abnormal blood pigments were observed spectroscopically and there was no clinical evidence of cyanosis. Any urinary deposits from these patients were examined for sulphonamides by quantitative chemical analysis. All these examinations (made on more than 40 patients) were negative.

In three cases in which analyses of blood and pleural fluids were made, the

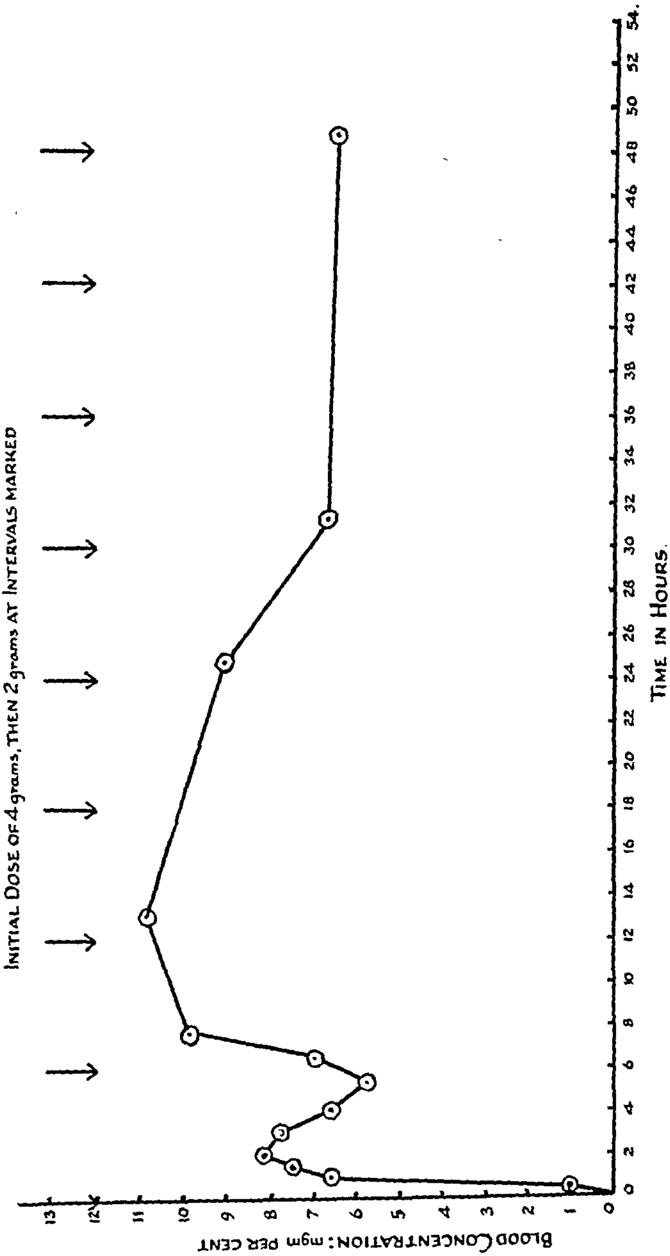


Fig. 7. Blood Concentrations on 4 Grams and 2 Grams 6 Hourly over 2 Days. Normal Patient

concentrations of sulphamethazine in the fluids were on the average about 10% higher than the blood levels.

When possible, simultaneous estimations in cerebrospinal fluid and blood were made, eleven specimens being obtained from seven patients. The cerebrospinal fluid level of free sulphamethazine varied from 46% to 119% of the blood level; the values most frequently obtained were of the order of 60%.

Taken all together, these results show that sulphamethazine is freely and rapidly absorbed by man and does not give rise to toxic symptoms or renal complications even when given in large doses.

SUMMARY AND CONCLUSIONS

1. The properties of *2*-*4'*-aminobenzenesulphonylamino-*4*:*6*-dimethylpyrimidine (sulphamethazine) are described. The solubilities of this compound and of its acetyl derivative in water are recorded over a range of pH values and are compared with similar data for sulphapyridine, sulphathiazole and sulphadiazine.
2. The *in vitro* action of sulphamethazine resembles that of sulphapyridine.
3. In experimental infections of mice with haemolytic streptococci and pneumococci, sulphapyridine and sulphamethazine gave very similar results. Against streptococci both compounds were more effective than sulphanilamide. Against pneumococci this difference was less marked.
4. Following a single oral dose to mice, the concentration in the blood rises more rapidly and reaches a higher level with sulphamethazine than with either sulphanilamide or sulphapyridine.
5. Following a single intravenous dose to mice, the concentration in the blood falls more slowly with sulphamethazine than with sulphanilamide or sulphapyridine.
6. The toxicities of sulphamethazine and its acetyl derivative to mice are discussed.
7. The results of absorption and excretion studies in man are reported and discussed.
8. It is concluded that sulphamethazine probably has advantages over the other sulphanilamides at present in use.

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NOBLE-COLLIP SHOCK: THERAPEUTIC EFFECTS WITH AUTONOMIC DEPRESSANTS; MOTION FACTORS

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INTRODUCTION

The type of shock[1] induced in normal and tumor-bearing mice by the use of injection of the O antigens of gram negative bacteria has previously been studied and reported (1) by us. The generally accepted view that such toxins are direct vascular poisons has been challenged by Gastinel and Reilly (2) on the basis of their observations that purified typhoid-paratyphoid antigens were several times more toxic when injected so as to be in direct contact with the splanchnic nerve than when administered intravenously. The suggestion that this type of damage may entail a nervous factor received further support from the experiments of Feldman and Gellhorn (3) who demonstrated that the injection of typhoid-paratyphoid vaccine led to a simultaneous stimulation of both the vago-insulin and sympathetico-adrenal systems.

These considerations raise a question as to the possible rôle in shock of disorganization of the autonomic nervous system and of the applicability of therapeutic measures directed specificially at this system. Heightened activity of the parasympathetic nervous system following trauma, burns, etc., would be expected to result in the excessive liberation of acetylcholine. Carlson (4) has stated that the symptoms of shock resemble those of acetylcholine intoxication. Also Chigura (5) and Nakamura (6) in their studies on anaphylactic shock found that acetylcholine was a more likely inciting agent than histamine, since the administration of physostigmine, which greatly prolongs the action of acetylcholine by inhibiting the acetylcholine-destroying enzyme, intensified the anaphylactic shock, and, conversely, the administration of atropine, which abolishes the response of smooth muscle and glands to acetylcholine, completely abolished anaphylactic shock.

In studying the prophylaxis and therapy of toxemic shock, it seemed desirable to make a comparative study utilizing a second and dissimilar type of shock. The Noble-Collip (7, 8) wheel technique was chosen because it appeared to offer a standardizable type of injury. Noble and Collip considered this injury to be traumatic shock. We have confirmed the experimental results of Noble and Collip, including their finding that the curve relating number of tumblings with percentage of deaths and with survival time is satisfactorily reproducible. By varying the number of falls to which the rat is subjected, good control is possible

[1] Penner and Bernheim (13) have recently reported that the injection of *Shigella* endotoxin produced most of the symptoms of the classical shock syndrome, and concluded that the intoxication corresponded to typical shock.

in inducing any desired degree of this type of injury; and the device thus lends itself to tests of the relative efficiency of certain therapeutic procedures.

EXPERIMENTAL. White male rats of a standardized strain weighing 150–170 grams were subjected to tumbling for various periods, using substantially the apparatus and technique described by Noble and Collip.

1. *Protection by atropine.* Of each pair used, one animal received pre-treatment with 8–10 mgm.[2] atropine, injected intravenously. The treated animals were placed in the wheel about thirty minutes after injection, by which time they

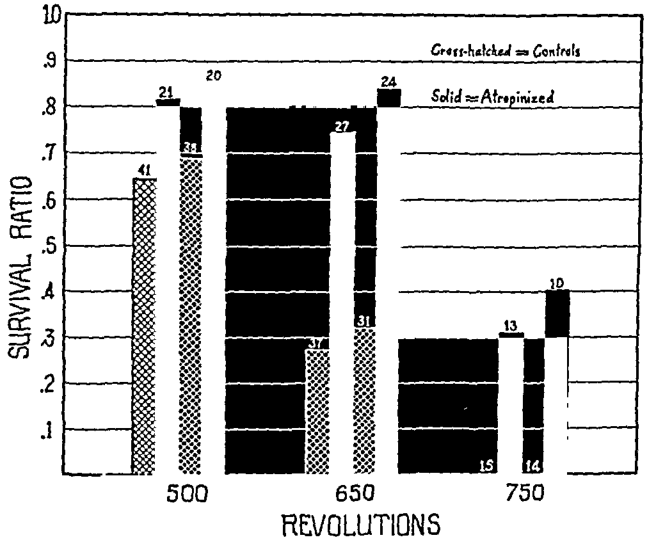

FIG. 1. EFFECT OF PRE-TREATMENT WITH ATROPINE ON THE RESISTANCE OF RATS TO NOBLE-COLLIP TUMBLING

Of each quadripartite group of columns, the two columns at the right represent the survival ratios of controls (cross-hatched), and the survival ratios of atropinized rats (solid), computed from the total data exclusive of animals exhibiting visceral hemorrhage at death. The two left-most columns of each group represent similar survival ratios computed from the total data inclusive of animals showing hemorrhage at death. From the segregation of the data thus, it may be seen that mesenteric hemorrhage induced during tumbling, and usually fatal, does not significantly alter the magnitude of the atropine effect. The number of animals used for each determination is indicated at the top of each column.

had recovered from early symptoms of hyperactivity. After removal from the wheel, the animals were carefully observed and any fatalities were autopsied. Survival for twenty-four hours was usually followed by complete recovery. The results, shown graphically in fig. 1, indicate that pre-treatment with atropine increased the survival ratios significantly at levels above 500 revolutions. Similar experiments using scopolamine gave equivalent results.

The difference in appearance and behavior between treated animals and con-

[2] This dosage is not excessive in view of the extreme tolerance of rats to atropine. Moreover, in other experiments, smaller doses exhibited a similar effect.

trols after tumbling in the Noble-Collip wheel was consistent with the survival data. Controls showed irregular spasmodic breathing, diarrhea and extreme prostration. Treated animals showed regular rapid breathing and little or no diarrhea. Within a short time most of the treated animals were moving about almost normally, and with the fur in good condition; the controls still were prostrate and with fur ruffled. Among fatalities death occurred between a few minutes and a few hours after removal from the wheel. At autopsy the controls exhibited the marked congestion of the viscera noted by Noble and Collip (see fig. 2). Treated animals, before and at the peak of the survival curves (see fig. 1), showed considerably less congestion.

2. *Sensitization by physostigmine.* Since physostigmine prolongs the action of acetylcholine, small doses of physostigmine should lower the resistance of the animals to Noble-Collip damage if this damage follows from the liberation of acetylcholine. This point was tested with animals which had received subcutaneous injections of 0.12 mgm. of physostigmine (a small fraction of the lethal dose) followed by tumbling in the Noble-Collip wheel. These animals (twelve) were all rendered sensitive, eight dying in the wheel at less than 400 revolutions, and four dying at less than 600 revolutions.

3. *Protection by central nervous depressants.* The effect of *light* anesthesia, without loss of consciousness, induced by intraperitoneal injection of pentobarbital (Nembutal) exerted a protective action against Noble-Collip trauma. Of the fifteen animals so treated seven survived and one died after 700 revolutions; six survived and one died after 1000 revolutions.

Of five animals in *deep* pentobarbital anesthesia, four did not survive 400 revolutions. This result is in agreement with the findings of Noble and Collip, and is not unexpected since the complete muscular relaxation of the unconscious animal permits mechanical tearing of the visceral mesenteries and death often occurs from hemorrhage.

4. *Protection by abdominal bandaging.* Abdominal bandaging designed to immoblize the viscera gave substantial protection against the effects of tumbling. The combination of bandaging and light anesthesia gave remarkable protection. Of eight animals so treated, seven showed satisfactory recovery from 1300 revolutions; one was alive at the end of 2000 revolutions, but died shortly thereafter. The favorable effect of abdominal bandaging was noted by Noble and Collip.

5. *Conditioning by previous tumbling.* In attempting the re-use a series of animals whose history had included survival from tumbling at 400–600 revolutions two weeks earlier, we subjected twenty rats to tumbling for a second time. These animals were found to exhibit noteworthy resistance to the second tumbling. Three of them survived and one died after 1900 revolutions; nine survived and one died after 1300 revolutions; six survived 1000 revolutions. On the whole such conditioned animals seemed to be between 50 and 100% more resistant than unconditioned animals of similar weight and age.

Another series of twelve animals was tumbled on successive days according to the following increasing schedule: 100, 200, 300, 400, 500, 600, 800, 1000, 1500 revolutions. Each tumbling appeared to increase the resistance of the animals

to subsequent tumbling. Of this series one animals withstood 3100 revolutions on the 10th day. Although the precise upper limit of this conditioned resistance

FIG. 2. VISCERAL CONGESTION TYPICAL OF ANIMALS TUMBLED IN THE NOBLE-COLLIP WHEEL

Both animals were subjected to 600 revolutions. Specimen at left was pre-treated with atropine. Note the relative absence of congestion. Specimen at the right was untreated. Note marked visceral congestion.

was not ascertained, the remaining animals survived 1500 revolutions. Control animals do not survive above 800 revolutions.

6. *Motion effects in Noble-Collip injury.* The effectiveness of atropine and of

light anesthesia, considered together with mechanical features of Noble-Collip tumbling, suggests the existence of an important component in Noble-Collip injury, distinct from traumatic damage. This component may be associated with rapid acceleration and deceleration acting primarily on the viscera and resulting in over-stimulation of the parasympathetic nervous system.

We attempted to differentiate this factor from general traumatic damage by subjecting rats to rapid up-and-down shaking in a mechanical device consisting of a box completely lined with sponge rubber and just large enough snugly to accommodate one rat. This box was supported horizontally on a lever driven by a crank arrangement. The box was made to oscillate up-and-down at four cycles per second, through a total distance of six inches. This provided violent acceleration and deceleration, but the animals, confined between rubber sponge, were thereby protected from gross trauma. The legs were not tied. The mean time to death of twelve untreated rats thus shaken was found to be 59 minutes, plus or minus not more than 15 minutes. It was found that the animals shaken to death in this device showed no bruises or lacerations and no mesenteric hemorrhage, but some visceral congestion. Those dying from Noble-Collip tumbling always showed marked visceral congestion, cutaneous bruises and lacerations, and often severe mesenteric hemorrhage. During the period of shaking the animals were removed periodically for examination. Diarrhea was an early and constant symptom, and prostration increased progressively until death. At no time, either in the shaking device or in the Noble-Collip wheel, was there any evidence of interference with the normal equilibrium pattern, i.e., conscious animals showed complete possession of their righting reaction.

Preliminary experiments (8 animals) with the shaking device indicated that atropine (administered as in Section 1) conferred some protection (mean time to death, 69 minutes). Light anesthesia with pentobarbital more than doubled the normal time to death in the shaking device of five animals. Eight animals conditioned (as in Section 5) in the Noble-Collip apparatus showed remarkable resistance to death in the shaking box, seven surviving two hours and one animal surviving four hours of continuous shaking. The converse of such conditioning was tested by shaking nine animals for 30 minutes on each of three successive days. Four days after the last shaking they were tumbled in the Noble-Collip wheel. All survived 1000 revolutions, indicating that conditioning by shaking and by Noble-Collip tumbling are reciprocally protective.

In view of the absence of trauma, hemorrhage, or gross damage, it seems reasonable to conclude that death in the shaking device resulted from continued and rapid acceleration and deceleration rather than from traumatic injury. The transfer of conditioning from tumbling to shaking and the converse, the protection afforded by light anesthesia against both tumbling and shaking, the partial protection afforded by atropine, and the presence of some visceral congestion due to shaking, all suggest that Noble-Collip injury is not wholly of traumatic origin.

7. *Other therapeutic and prophylactic measures.* In a series of exploratory

tests, rats were treated with the following materials both before and after tumbling in the Noble-Collip wheel:

	NUMBER OF ANIMALS	DOSAGE RANGE
Amino acids (cysteine, methionine, glycine) in various combinations....	2 each	2–7 mgm. each, subc.
Ascorbic acid........................	12	25–100 mgm. intraper. and subc.
Atropine plus ascorbic acid..........	2	as indicated
Atropine plus ergotamine.............	2	as indicated
Atropine plus vitamins of the B complex (pantothenate, thiamine, riboflavin)........................	2 each	as indicated
Calcium gluconate..................	2	0.25–.50 cc. 10% aq. intraven.
Calcium gluconate plus ascorbic acid	2	as indicated
Choline hydrochloride...............	2	2–7 mgm. subc.
Citrus pectin........................	12	0.5 cc. 2% aq. intraven.
Curare..............................	2	0.05–.3 mgm. subc.
Dextrose............................	2	0.25–.50 cc. 50% aq. intraven.
Epinephrine.........................	4	0.1–1.0 mgm. subc.
Ergotamine tartrate	2	0.5–1.5 mgm. intraper.
Liver concentrate (Lederle)..........	2	1.5 Units intraper.
Pantothenate........................	2	0.5–1.0 mgm. subc.
Riboflavin..........................	2	0.5–2.0 mgm. subc.
Yeast extract (Difco)................	2	Oral ad lib.
Yeast nucleic acid..................	2	3–7 mgm. subc.

With the possible exception of ascorbic acid, none of these treatments disposed the animal to greater resistance to tumbling. In view of the lack of positive results obtained with the other materials, the hypotheses which suggested their use need not be elaborated. Ascorbic acid, in large doses, and in a series of twelve animals, seemed to impart some added resistance to tumbling, although these experiments did not provide a quantitative estimate of this effect. This may be consistent with the report by Ungar (9) who found that ascorbic acid protected guinea pigs and rats against traumatic shock produced by falling weights.

Discussion. McEachern and coworkers (10) note that the mainstays in the treatment of seasickness, the classical form of motion sickness, are drugs of the atropine series and light anesthetics. These were the measures found effective in minimizing Noble-Collip injury. The protective effects of abdominal binding and of previous conditioning are additional features common to both Noble-Collip injury and motion sickness. McEachern and coworkers further state that vagotonia may be an important element in motion sickness, and that the classical evidence of surgical shock may be present.

We have attempted experimentally to simulate the acceleration-deceleration component of the complex motion inducing seasickness and airsickness. Our results with the shaking machine show that repeated accelerations and decelerations may cause death without gross mechanical trauma, and that the time

to death is broadly in the same order of magnitude as that for animals dying from Noble-Collip injury. In view of the demonstrated ability of shaking to produce death without the participation of the mechanical trauma found in Noble-Collip tumbling, and in view of the similarities in effective treatment of Noble-Collip injury, of injury due to shaking, and of classical motion sickness, it seems reasonable to conclude that motion sickness may be an important causative factor in the death of animals traumatized in the Noble-Collip apparatus.

The use of the term "motion sickness" as applied to rats treated in the shaking device is not intended to imply identity of this condition with seasickness or airsickness. The type of motion in the shaking device is only roughly comparable to that evoking the more common form of motion sickness among humans. Also, the common symptoms of the latter type of motion sickness (i.e., excessive salivation, peripheral vascular changes, etc.) were not obvious in the rats subjected to shaking. However, these qualifications do not preclude the possibility that the various types of motion sickness have certain elements of causation in common; especially so since the positive evidence (i.e., similarities in therapy and response to conditioning previously pointed out) for such an hypothesis seem reasonably convincing.

There is extensive evidence that the administration of anesthetics to a patient already in shock is likely to be disastrous, and that animals under anesthesia subjected to trauma exhibit increased susceptibility to shock (*vide* Noble and Collip). An adequate study does not appear to have been made of the combination of a drug of the atropine series and an appropriate anesthetic. This combination of centrally acting anesthetics together with parasympathetic inhibitors might have value in the treatment, and particularly in the prophylaxis of shock; and, in this connection, the report of Nosworthy (11) is suggestive. He asserts that the combination of scopolamine and morphine allows the use of much smaller doses of morphine, resulting in less respiratory depression; and he further reports that this procedure as part of an anti-shock program helped convert bad surgical risks into fair ones.

In view of the present military importance of motion sickness (12) and the feasibility of using the rat as an experimental animal, further exploration of the motion sickness component by the use of either tumbling or shaking procedures would seem to be indicated.

SUMMARY

Atropine, scopolamine, light anesthesia, abdominal binding, and conditioning by previous tumbling were treatments found to be effective in protecting rats against lethal damage induced by tumbling in the Noble-Collip apparatus. Physostigmine sensitized animals to Noble-Collip damage. These and other therapeutic data emphasize the involvement of cholinergic components of the autonomic nervous system in such damage.

The involvement of the parasympathetic nervous system, considered in relation to factors involved in the violent tumbling characteristic of the Noble-Collip procedure, suggests that the acceleration-deceleration effect is an important

component of Noble-Collip injury. Confirmation of this view was obtained by subjecting rats to rapid up-and-down shaking in a device designed to eliminate the type of gross trauma induced by Noble-Collip tumbling. Measures found effective in protecting rats from damage due to tumbling were also effective against shaking.

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THE EFFECT OF SODIUM CITRATE ADMINISTRATION ON EXCRETION OF LEAD IN URINE AND FECES

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Previous experimental and clinical studies of sodium citrate administration in lead poisoning (1)(2)(3) demonstrated a highly significant reduction in blood lead concentration with rapid amelioration of symptoms during the administration of this drug. It is important to the proper understanding of the therapeutic action of sodium citrate to learn whether lead disappears due to (*a*) deposition in tissue and bones or (*b*) increased excretion. Preliminary studies of lead excretion in the urine gave variable results although a trend toward increased excretion was observed. Experiments to determine the fate of the lead which disappeared from the blood stream are reported in this paper. In these experiments total urinary and fecal lead excretion was measured before and during the administration of sodium citrate to nine individuals who were suffering from the effects of chronic exposure to lead.

EXPERIMENTAL. Patients selected for study had been exposed to industrial lead hazards for periods ranging from several months to several years. Eight of the nine patients exhibited definite evidence of lead poisoning. Clinical histories of these have been reported elsewhere (2). The remaining patient (R. R.), presented no definite signs of lead intoxication but had worked as a painter for many years.

After a preliminary period of hospitalization to eliminate effects of medication that some patients had received before admission, and to permit adjustment to hospital conditions, the collection of excreta was begun. All urine voided was collected in specially cleaned bottles containing 5 cc. of lead-free toluene as a preservative. Feces were collected in stainless steel bed pans and immediately transferred to waxed paper cartons. Precautions were taken throughout to avoid additions or losses of lead.

The experiments were divided into two stages: a control period, during which the patients received no medication known to influence lead metabolism, and a period of citrate therapy during which sodium citrate 5 grams three times daily was administered by mouth (a few patients received 20 grams daily). Throughout the study all patients received the standard house diet. In a few cases where constipation was present to a degree which might have interfered with the collection of feces, patients received 0 3 to 0 6 gram of cascara sagrada daily throughout the entire study.

Urine and feces were collected during 4 to 6 day periods and aliquot specimens were analyzed for lead by the method of Letonoff and Reinhold (4), slightly modified (5). Preparation of feces for sampling was facilitated by the addition of 5 cc. of lead-free hydrochloric acid to the total sample of feces and heating to dryness on a steam bath with frequent stirring. This procedure yielded a thoroughly mixed product from which representative samples were easily obtained after crushing in a mortar. The 24 hour excretion of lead in urine and feces for each period was calculated from these analyses.

RESULTS AND DISCUSSION. The results are presented graphically in figure 1. With one exception (J. W.) urinary lead excretion was increased during citrate

administration. Elimination in the feces and total output of lead were also increased during this period except in one case (J. N.).. This patient had received citrate therapy during a 3 day period 4 days before the control period. The mean increase in total lead excretion during citrate administration was 0.652 ± 0.271 mgm. per 24 hours. This is statistically significant (p = 0.05 by Fisher's t Test (6)). Mean increases in daily urinary and fecal lead excretion of 0.108 ± 0.053 mgm. and 0.544 ± 0.251 mgm. respectively, during citrate therapy (p = 0.08 and 0.07) approach but do not quite attain statistical signif-

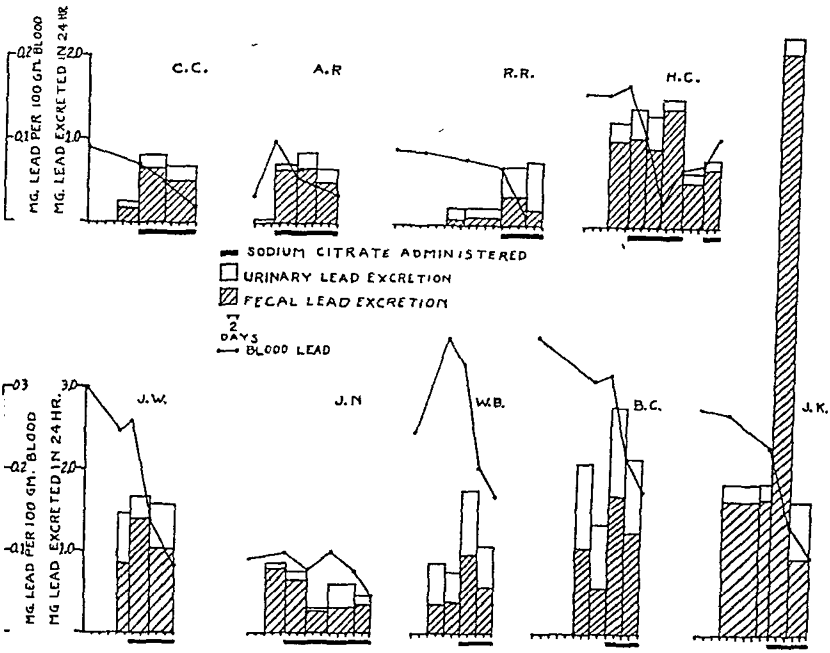

FIG. 1. CHANGES IN THE URINARY AND FECAL LEAD EXCRETION IN 9 PATIENTS WITH CHRONIC EXPOSURE TO LEAD, BEFORE AND DURING THE ADMINISTRATION OF SODIUM CITRATE

icance. While the increased lead excretion is mainly by way of the feces, a few patients show greater increase in urinary excretion. Greater individual variations explain the failure to demonstrate statistically significant increase in excretion in urine and feces considered separately, while the total excretion was significantly increased. After a marked initial increase in lead excretion during citrate administration, a downward trend in lead output is apparent. It was not possible to evaluate the duration of the action of citrate or the effects of subsequent courses of therapy because of unwillingness of our subjects to coöperate for longer periods. A statistically significant drop in blood lead was found coinci-

dent with the increased lead elimination. Blood lead figures have been reported elsewhere for 8 of the 9 patients (2).

No change in excretion of calcium or phosphorus in urine and feces was found during citrate administration in three subjects studied. The lead lost by increased excretion in feces and urine greatly exceeded the amount disappearing from the circulation as calculated from decrease in blood lead concentration.

It is of interest that Donnelly and Holman (7) have recently found sodium citrate to ameliorate the effects of uranium, a metal similar in many ways to lead in its action on the body.

SUMMARY

The excretion of lead in urine and feces of 9 patients after long-continued exposure to lead was determined before and during the administration of sodium citrate. Appreciable increases in urinary, fecal and total excretion in 8 of the 9 cases occurred during citrate therapy. The mean increase in total excretion of lead during citrate therapy for all 9 cases is statistically significant.

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FORMATION OF METHEMOGLOBIN

I. SPECIES' DIFFERENCES WITH ACETANILIDE AND ACETOPHENETIDINE

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Szigeti (1) in 1893 pointed out that among different laboratory animals there was a marked species difference in the formation of methemoglobin following the administration of aniline derivatives. Heubner (2) confirmed these differences with a wide variety of compounds. The work of both of these investigators was mainly qualitative; it indicated in general that with these drugs the carnivorous animals form methemoglobin readily and the herbivorous poorly. A number of studies have also been made of the comparative methemoglobin-forming powers of various drugs when administered to one species of animal (2–7) but there has been no systematic study of the magnitude of the response by different species to any one drug.

In the investigation reported here, the maximum amounts of methemoglobin produced by single doses of acetanilide and acetophenetidine in human beings, cats, dogs, rats, monkeys and rabbits were determined. These determinations were preliminary to a study of possible species differences in the intermediary metabolism of aniline derivatives.

The formation of methemoglobin, which in certain animals is a striking effect of these drugs, is often regarded as an important feature in their toxicity. This belief, however, is not supported by any direct evidence; the comparative toxicities for single and repeated doses are not in the same order of sensitivity to methemoglobin formation as found here (8, 9). The monkey and rabbit form little or no methemoglobin, even from lethal doses of acetanilide, while the cat and man may form considerable amounts from doses that have no appreciable toxic effect. With blood *in vitro* there is no species difference in the formation of methemoglobin from the presumed metabolites of these drugs (10–17). Therefore, the species differences in methemoglobin formation, as shown in this study, are taken by the author to indicate either a different intermediary metabolism (2) of the antipyretics in different animals or a great difference in the rate of reconversion of methemoglobin. If either condition holds, it follows that the results obtained from experiments on laboratory animals with these antipyretics cannot be applied to man unless it can be shown that both conditions are the same in the animal as in man.

Experimental Procedure. Acetanilide and acetophenetidine were administered to the experimental animals in 2% gum acacia suspension by stomach tube; no food was given for 16 hours before and 5 hours after the administration. The drugs were given to the human subjects in tablets which were swallowed on an empty stomach. Blood was drawn at

1 hour intervals following the administration and the total hemoglobin, methemoglobin and sulfhemoglobin determined in 0.1 cc. samples of blood after the procedure of Evelyn and Malloy (18) which is accurate to ±0.2 g. of blood pigment per 100 cc. of blood. A curve was plotted for the amount of methemoglobin in relation to the time following administration of the drugs and the maximum methemoglobin formation was taken from this curve. A typical curve is given in Paper II of this series (19).

In blood freshly drawn from the animal and man before the drugs were administered, the amounts of methemoglobin and sulfhemoglobin in no instance exceeded that of the experimental error of the method used for determination, ±0.2 g. When the blood was allowed to stand for 3 or 4 hours, however, appreciable amounts of the pigment were occasionally found; this formation could be slowed by adding the blood sample to the phosphate buffer containing toluene.

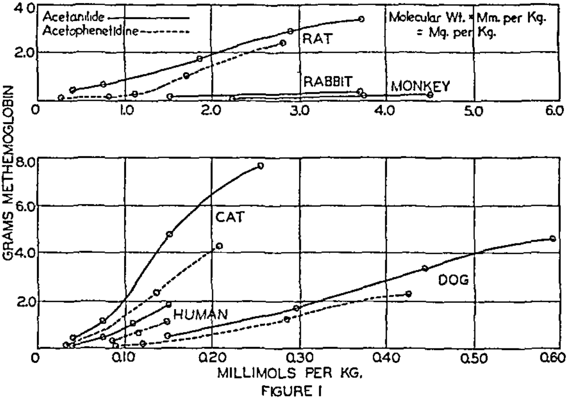

FIG. 1. SPECIES DIFFERENCES AT MAXIMUM FORMATION OF METHEMOGLOBIN FOR VARIOUS DOSES OF ACETANILIDE AND ACETOPHENETIDINE

EXPERIMENTAL RESULTS. The average maximum amounts of methemoglobin formed after single doses of acetanilide and acetophenetidine are shown in figure 1.

Acetanilide, even on a molar basis for dose, is more active in forming methemoglobin than is acetophenetidine; for the cat and man, the comparative activity for the doses used varied between 1:1.5 and 1:1.9 with an average of 1:1.7; for the dog and rat, 1:1.2 to 1:1.7 with an average of 1:1.4.

The curves of figure 1 are S-shaped. The general indications are: *1* Methemoglobin is formed only when certain minimal doses of acetanilide and acetophenetidine are exceeded. This minimal dose, to be referred to here as the

threshold dose, varies with different animals. *2* The amount of methemoglobin formed does not increase in direct proportion to the increase in dose (3, 20, 21). *3* There is an upper limit to the formation of methemoglobin irrespective of the dose (3, 10, 22, 23). Administration of amounts beyond that producing the maximum concentration of methemoglobin only increases the length of time the methemoglobin persists.

From results obtained here on the rabbit and monkey, these animals may be considered as virtually non-responsive since doses as large as 3.70 or 4.50 millimols per kg. result in the formation of less than 0.5 g. of methemoglobin. Smith (24) found no significant amount of methemoglobin in monkeys given single doses of acetanilide. Kruse and McEllroy, Heubner, and Young and Wilson (2, 25, 26) have obtained similar results on rabbits.

For the remaining animals and man, the threshold dose was taken arbitrarily as that amount of acetanilide or acetophenetidine which would produce an average maximum of 0.4 g. of methemoglobin, which is twice the maximum

TABLE 1

Comparative species sensitivity to formation of methemoglobin: cat taken as 100

FOR DOSES REQUIRED TO PRODUCE METHEMOGLOBIN	ACETANILIDE				ACETOPHENETIDINE			
	Cat	Man	Dog	Rat	Cat	Man	Dog	Rat
g.								
0.5	100	53	27	6	100	63	32	5
1.0	100	56	30	5	100	62	35	6
1.5	100	56	28	5	100		39	5
2.0	100	60	30	5	100		36	5
2.5	100		30	4	100		32	5
3.0	100		30	4				

analytical error and corresponds to 2.7% of methemoglobin at a total hemoglobin of 15 g. In man the threshold dose for acetanilide was taken as 0.065 millimols per kg. and for acetophenetidine 0.084 millimols per kg. The variations for these doses are given in a later section. These doses correspond respectively to approximately 8½ and 15 grains for a man of average size.

The comparative sensitivity of the cat, man, dog and rat to the formation of methemoglobin for amounts of acetanilide and acetophenetidine required to produce 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g. of methemoglobin is shown in table 1. Up to the point of marked flattening in the curves (fig. 1), the relation between the different animals is maintained fairly constant and is about the same for the two drugs. In approximate relation, man is slightly more than half as sensitive as the cat, the dog half as sensitive as man, and the rat one-sixth as sensitive as the dog.

In no instance after the administration of acetanilide or acetophenetidine was sulfhemoglobin found in an amount in excess of that of the maximum analytical error, ± 0.2 g.

The Cat. There are no records in the literature of the maximum amount of methemoglobin formed by the cat after acetanilide and acetophenetidine are administered orally. Ellinger (10) injected acetanilide intravenously into 3 cats in doses of 0.037, 0.35 and 0.40 millimols per kg. and found in each instance that 36 to 38% of the hemoglobin was converted into methemoglobin. In another cat given 1.24 millimols per kg. over a period of 140 minutes, 36% was also converted. It is probable that acetanilide is changed to an active oxidant (in relation to hemoglobin) through the action of the liver (27); therefore it is possible that the formation of methemoglobin occurs more rapidly from acetanilide reaching the liver directly in the portal circulation than that reaching it only in the systemic circulation. The difference between the findings reported in this paper and those of Ellinger suggest that with these drugs, consideration must be given not only to species differences but also to the method of administration (28). They are rarely, in therapy, administered intravenously to man.

In the experiments reported here, 16 cats were used with a total of 46 determinations of the maximum formation of methemoglobin. The total hemoglobin of the cats ranged from 8.9 to 12.9 grams with an average of 10.7 grams. With acetanilide, doses of 0.037, 0.074, 0.15 and 0.26 millimols per kg. were given; the minimum, maximum and average grams of methemoglobin formed by these doses were, respectively: 0.5, 1.0, and 0.6; 0.5, 2.3 and 1.1; 3.2, 6.4 and 4.7; and 5.2, 9.0 and 7.4. With acetophenetidine, doses of 0.056, 0.14 and 0.22 millimols per kg. were given; the minimum, maximum and average grams of methemoglobin formed were respectively: 0.0, 0.4 and 0.1; 0.1, 4.3 and 2.2; and 3.7, 5.1 and 4.3.

Man. Sixty-nine subjects were used with a total of 222 determinations of the maximum formation of methemoglobin. The total hemoglobin ranged from 8.2 to 16.0 grams with an averate of 13.9. With acetanilide, the doses given were 0.037, 0.074, 0.11 and 0.15 millimols per kg. The minimum, maximum and average grams of methemoglobin formed by these doses were respectively: 0.0, 0.2 and 0.1; 0.1, 0.9 and 0.5 g.; 0.6, 1.8 and 1.1; and 1.6, 2.2 and 2.0. With acetophenetidine, the doses were 0.056, 0.084, 0.11 and 0.14 millimols per kg. The minimum, maximum and average grams of methemoglobin found were respectively: 0.0, 0.2 and 0.1; 0.0, 0.8 and 0.4; 0.1, 0.9 and 0.6; and 0.7, 1.3 and 1.1.

The Dog. Young and Wilson (25) were unable to detect methemoglobin in the blood of dogs given acetanilide in alcohol intravenously. Dennig (22) found 42% of the hemoglobin converted to methemoglobin after administering 2.3 millimols per kg. of acetophenetidine and 60% after 4.5 to 10.0 millimols per kg. of acetanilide; both drugs were given with the food. In one dog to which acetanilid was given by mouth, Payne (28) found 50% of the hemoglobin converted to methemoglobin after a dose of 1.44 millimols per kg., 52% after 1.8 millimols kg. and 62% after 4.9 millimols per kg.; and in another, 38% after 1.76 millimols per kg., 51% after 2.2 millimols per kg. and 71% after 5.44 millimols per kg. There is no evidence that these values are maximal.

In the series of experiments reported here, 6 dogs were used with a total of 34 determinations. The total hemoglobin ranged from 9.7 to 16.9 grams with an

average of 14.2. With acetanilide, the amounts given were 0.15, 0.3, 0.45 and 0.6 millimols per kg. The minimum, maximum and average grams of methemoglobin formed were respectively: 0.3, 0.9 and 0.6; 1.2, 2.7 and 1.9; 1.7, 5.7 and 3.7; and 2.6, 6.5 and 4.5. With acetophenetidine, the amounts given were 0.084, 0.17, 0.28 and 0.42 millimols per kg. The minimum, maximum and average grams of methemoglobin formed were respectively: 0.0, 0.2 and 0.1; 0.0, 0.9 and 0.4; 0.2, 3.3 and 1.5; and 0.5, 5.0 and 2.2.

The Rat. In post-absorptive rats given acetanilide in doses of 7.4 millimols per kg., Smith (24) found an average of 4.0 grams of methemoglobin 4 hours after administration.

In the experiments reported here, 71 animals were used with a total of 71 determinations. The total hemoglobin varied from 9.9 to 17.4 grams with an average of 14.2. The doses of acetanilide administered were 0.37, 0.74, 1.85, 2.96 and 3.70 millimols per kg. The minimum, maximum and average grams of methemoglobin formed were respectively: 0.3, 0.7 and 0.4; 0.1, 0.9 and 0.6; 0.3, 2.7 and 1.9; 0.9, 4.8 and 3.1; and 1.9, 4.0 and 3.4. The amounts of acetophenetidine given were 0.28, 0.84, 1.11, 1.68, 2.80 millimols per kg. The minimum, maximum and average grams of methemoglobin formed were respectively: 0.0, 0.2 and 0.1; 0.0, 0.0 and 0.0; 0.0, 1.0 and 0.3; 0.1, 3.6 and 1.6; and 0.5, 4.9 and 2.5.

It is possible that with the large amounts of the drugs given to rats, the time required for absorption may prolong the formation of methemoglobin at the expense of some rise in the amount formed.

CONCLUSIONS

1. Acetanilide, even on a molar basis of dose, is more active in forming methemoglobin than is acetophenetidine.

2. The curve obtained by plotting methemoglobin against dose of drug administered is S-shaped. Methemoglobin is formed only when a certain minimal dose is exceeded and there is an upper limit to the formation irrespective of dose.

3. The rabbit and monkey form virtually no methemoglobin with acetanilide and acetophenetidine. In order of sensitivity to methemoglobin formation from these drugs, man is slightly more than half as sensitive as the cat, the dog half as sensitive as man, and the rat one-sixth as sensitive as the dog. These differences are taken to indicate possible differences in the metabolism of these drugs.

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FORMATION OF METHEMOGLOBIN

II. REPEATED ADMINISTRATION OF ACETANILIDE AND ACETOPHENETIDINE

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There is no evidence in the literature that methemoglobin is accumulative in man from repeated administrations of acetanilide (1) or acetophenetidine in ordinary therapeutic doses. The possibility exists, however, that if the dose is large a progressive rise may occur. The possibility likewise exists that methemoglobin may be converted to the more stable sulfhemoglobin and that this pigment may accumulate.

Smith (2) gave monkeys acetanilide in daily doses of 1 millimol per kg. for 95 days and the largest amount of methemoglobin found was 0.6 gram. When the daily dose was increased to 4 millimols per kg. and continued for 104 days, the largest amounts of methemoglobin and sulfhemoglobin found were respectively 1.0 and 0.4 gram; the amounts fell during the last 30 to 40 days of the experiment. In a similar series of experiments, Smith (2) gave rats 4 millimols per kg. of acetanilide daily for 30 days and the largest amounts of methemoglobin and sulfhemoglobin were 1.8 grams. When acetophenetidine was similarly administered and in the same dose, the largest amount of methemoglobin was 1.8 grams and of sulfhemoglobin, 0.8 gram.

The choice of the monkey and rat for this study was unfortunate because of their insensitivity (3) to the drugs. With the large doses used, considerable time would be required for absorption and elimination, so that the action of the drugs would be prolonged with persistence of the methemoglobin. In the cat and man, in which small amounts of the drug are required to produce methemoglobin, there are correspondingly more rapid elimination of the drugs and disappearance of methemoglobin. As much as 6 grams of methemoglobin produced by a single dose of 0.20 millimols per kg. of acetanilide will disappear from the blood of the cat in less than 24 hours. In man, the 0.6 to 1.8 grams of methemoglobin produced by 0.11 millimols per kg. of acetanilide will disappear from the blood in 3 to 10 hours.

The production and disappearance of methemoglobin in a human subject receiving single and repeated doses of acetanilide and acetophenetidine is shown in figure 1. This subject was an adult male of 71 kg. who was somewhat above average in his sensitivity to the formation of methemoglobin (3). The data given as A, figure 1, were obtained when he was given 3 grams of acetophenetidine in 3 doses of 1 gram (0.084 millimols per kg.) at intervals of approximately 4 hours. Within 1 to 2 hours after each dose the methemoglobin rose to a maximum of 0.7 to 0.75 gram, but disappeared from the blood within the 4

hour intervals between the doses; there was therefore no progressive rise with the repeated doses.

Curve B of figure 1 was obtained when 0.5 gram (0.052 millimols) of acetanilide was given as a single dose. The methemoglobin rose to a maximum of 0.69 gram at $3\frac{1}{2}$ hours and thereafter declined slowly, disappearing only after 8 hours following the administration of the drug. For single doses of acetanilide and acetophenetidine which give approximately the same maximum amounts of

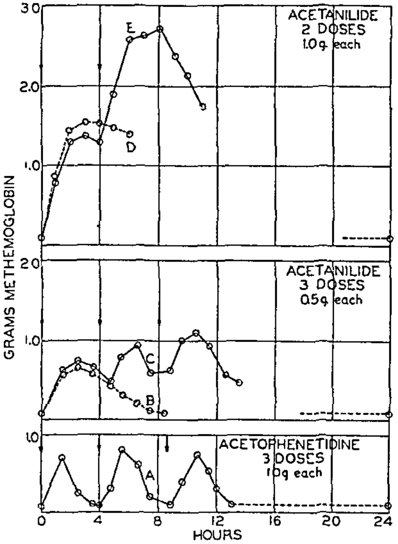

FIG. 1

methemoglobin (*A* and *B*, fig. 1), that from acetanilide disappears much more slowly than that from acetophenetidine. This prolongation may possibly be due to a much slower absorption of acetanilide, in which event its relative strength in forming methemoglobin would be much greater than indicated by the doses of the two drugs; or it may be due to differences in the intermediary metabolism of the drugs, and as such may indicate a more prolonged therapeutic action from acetanilide. When acetanilide was given in doses of 0.5 gram at intervals of 4 hours (Curve *C*, figure 1) some methemoglobin from one dose persisted at the

time of the next and there was an additive effect corresponding to the amount persisting. Thus as seen in Curve *C*, figure 1, the maximum after the first dose was 0.75 gram, after the second dose, 0.95 gram; and after the third, 1.1 gram. Curve D, figure 1 was obtained following a single dose of 1 gram (0.10 millimols per kg.) of acetanilide; the maximum methemoglobin was reached at 4 hours at 1.51 grams; at 6½ hours it had fallen to only 1.4 grams. When two doses were taken 4 hours apart (Curve *E*, figure 1) the maximum was 2.75 grams—a nearly direct addition.

In the particular subject used, it would appear that acetophenetidine in doses of 1 gram at intervals of 4 hours led to no accumulation of methemoglobin within

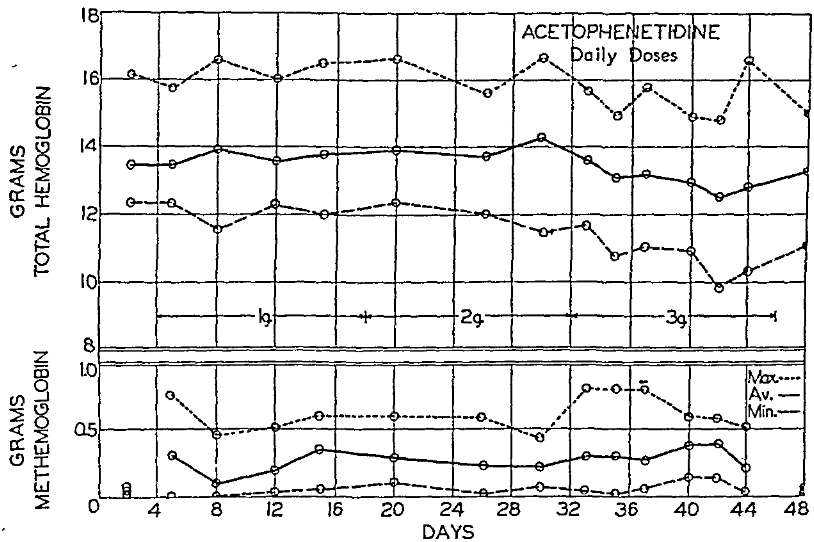

Fig. 2

the day, but that doses of acetanilide of 0.5 gram and above, given at these intervals, led to progressive rise in methemoglobin.

In all subjects used (3) methemoglobin, regardless of the amounts formed, disappeared from the blood within 18 hours and it was therefore improbable that the administration of acetanilide or acetophenetidine on one day would have any additive effect on that produced from the continuation of the medication on subsequent days. Since this feature, however, constitutes the essential one of the accumulation, it was investigated on a group of subjects of significant size: 15 male and 14 female subjects. In the first series of experiments each subject, during the first two weeks was given before breakfast 1 gram of acetophenetidine; during the third and fourth weeks, 2 grams daily in 2 doses, morning and noon; and during the fifth and sixth weeks, 3 grams daily in 3 doses, morning, noon and

6 o'clock. Methemoglobin, sulfhemoglobin, and total hemoglobin were determined 3 times a week, 2 to 3 hours after the morning dose and hence approximately at the time of the maximum formation following this dose. The results obtained are shown in figure 2. At the first determination, the methemoglobin averaged 0.3 gram with extremes of 0.0 and 0.75. On no occasion during the experiment was there an appreciable rise above these amounts. No sulfhemoglobin was found in excess of the maximum error of the analytical method (3). There was no decrease in total hemoglobin during the time 1 and 2 grams of acetophenetidine were administered daily; there were some indications of a slight drop during the two weeks in which 3 grams were administered daily.

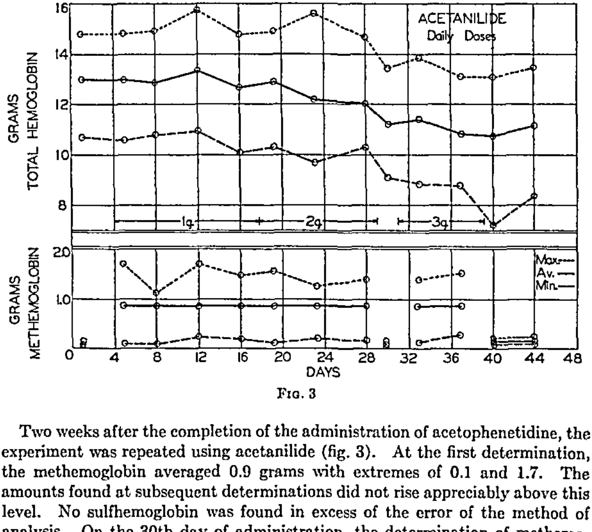

FIG. 3

Two weeks after the completion of the administration of acetophenetidine, the experiment was repeated using acetanilide (fig. 3). At the first determination, the methemoglobin averaged 0.9 grams with extremes of 0.1 and 1.7. The amounts found at subsequent determinations did not rise appreciably above this level. No sulfhemoglobin was found in excess of the error of the method of analysis. On the 30th day of administration, the determination of methemoglobin was made in the morning prior to the first administration of the acetanilide for the day; the methemoblogin was at the normal level. About one-third of the subjects developed definite cyanosis when receiving 3 grams of acetanilide daily. No determinations of methemoglobin were made after successive doses in a single day but judging from Curve E, Figure 1, the daily accumulation after the third dose may, for some subjects, have been well in excess of 3 grams.

CONCLUSIONS

1. Acetophenetidine given to human subjects in 3 doses of 1 gram each per day produces no additive rise in methemoglobin within the day. There was no accumulative formation of methemoglobin or sulfhemoglobin when 1, 2 and 3 grams were given daily for 6 weeks.

2. Acetanilide given in 3 doses of 0.5 gram each per day caused only a slight additive rise in methemoglobin within the day but a marked rise when given in doses of 1 gram. There was no accumulative formation of methemoglobin when 1, 2 and 3 grams were given daily for 5 weeks.

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STUDIES ON SULFONAMIDE-RESISTANT ORGANISMS

III. ON THE ORIGIN OF SULFONAMIDE-RESISTANT PNEUMOCOCCI

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Sulfonamide-resistant strains of pneumococcus and other microorganisms are formed readily by repeated or continued exposure of sensitive strains to the action of sulfonamide drugs. This has been demonstrated repeatedly in experimental studies. Little is known, however, of the origin of the resistant organisms composing the resistant strains. Whether these resistant bacteria are formed in small numbers during normal growth of any sensitive strain and are merely "bred-out" when such a strain is exposed to a sulfonamide, or whether they are produced only as a response to some action of the sulfonamide on the sensitive bacteria has not yet been determined, but as stated previously (1), there is some indirect evidence to support each of these possibilities.

In order to obtain more exact information on the origin of sulfonamide-resistant pneumococci, a quantitative study was made of the sensitivities of the individual organisms composing a sensitive strain of pneumococcus and the changes which occurred during the conversion of this sensitive strain to a highly resistant strain. The results of this study indicated that, at least in this case, the resistant pneumococci were produced in response to some action of the sulfonamide. That this also occurs in other instances was indicated by the results of a comparative study of the sensitivities of pneumococci composing other sensitive and resistant strains. This study showed that the most resistant organisms in the sensitive strains were significantly less resistant to sulfapyridine than the organisms of the corresponding resistant strains. The details of these studies are presented here.

I. CHANGES IN SULFAPYRIDINE SENSITIVITY DURING CONVERSION OF A SENSITIVE TO A RESISTANT STRAIN. A sensitive strain of pneumococcus was made highly resistant to sulfapyridine by serial transfer through media containing increasing concentrations of this drug. The sulfapyridine sensitivities of the organisms in the sensitive strain were determined by plating suitable dilutions of the parent culture in blood agar containing sulfapyridine, the numbers and sizes of colonies developing in various drug concentrations being used as indices of sensitivity. This determination of sensitivity was repeated at each step in the development of resistance, i.e., at each successive transfer.

METHODS. *Organism.* The pneumococcus used in this study was the highly sensitive *type II CH* strain This organism had never been exposed to the sulfonamides, having been isolated from a case of pneumococcal pneumonia prior to the advent of therapy with these drugs. For four years prior to the present study this strain had been passed through mice either daily or on alternate days. Prior to the initial transfer through media containing

sulfapyridine, a mouse passage culture was plated on blood agar; a single colony was selected from this plate and grown for 12 hours in beef heart infusion broth (2) enriched with 2% rabbit blood. This culture provided the parent organisms for the initial inoculum and test of sulfapyridine sensitivity.

Development of resistance. The procedure for preparing the resistant strain was essentially the same as that used previously (1); however, in order to clarify the presentation of results, the principal features of the method are described here briefly. The culture of the colony mentioned above was diluted serially in beef heart broth (2), and 0.5 cc. quantities of a 10^{-4} dilution were added to 50 cc. portions of beef heart medium containing 0, 1.25, 2.5 and 5 mgm. % sulfapyridine. (The details of preparation and enrichment of this medium have been described elsewhere (1).) These cultures were incubated at 37.5°C.; at various intervals samples were withdrawn and the number of pneumococci therein determined by means of plate counts. At the end of 48 hours incubation a transfer was made from the culture containing 2.5 mgm. % sulfapyridine (the highest concentration of drug permitting growth at this time) to beef heart broth containing no drug. This subculture was incubated for 12 hours, and was used as the source of organisms for a second experiment identical with the first except that the medium contained higher concentrations of the sulfonamide. This procedure was repeated until good growth was obtained in a medium containing 160 mgm. % sulfapyridine.

Measurement of sulfapyridine sensitivity: (a) Medium. The basal medium for this test was prepared by dissolving 2% agar in beef heart infusion broth, pH 7.8 (2). Various amounts of sulfapyridine were dissolved in this medium, which was then tubed in 13.5 cc quantities and sterilized by autoclaving. One tube of this agar, 0.5 cc. of freshly drawn defibrinated rabbit blood and 1.0 cc. of diluted culture were used in making each pour plate. The final concentrations of sulfapyridine in the various plates were 0, 0.3, 0.6, 1.25, 2.5, 5, 10, 20, 40, 80 and 144 mgm. %.

(*b*) *Technique of test.* At the beginning of the experiment, 1.0 cc. quantities of 10^{-5}, 10^{-6} and 10^{-7} dilutions of the colony culture were plated in agar containing 0, 0.3, 0.6, 1.25, 2.5 and 5 mgm. % sulfapyridine, and 1.0 cc. quantities of 10^{-3} and 10^{-4} dilutions were plated in agar containing 5, 10, 20, 40 and 80 mgm. % of the drug. At each subsequent stage in the preparation of the resistant strain, 10^{-5}, 10^{-6} and 10^{-7} dilutions of the subculture used in inoculating the drug broth were plated in sulfapyridine agar. Except where otherwise indicated, the colony counts and estimations of colony size recorded in the tables were made after the plates were incubated 24 hours. At this time the colonies on the control plates were well grown. Colony counts and estimates of size were also made after 48 hours of incubation; however, in nearly all instances, small and minute colonies were visible on drug-containing plates incubated 48 hours that were not evident on 24-hour plates. Since the late appearance of small colonies on the sulfapyridine plates may have been due to development of sulfonamide resistance, the 48 hour counts have been considered less representative of the original status of the organisms than those made after 24 hours incubation.

Control. In order to determine whether prolonged growth in the control medium affected sulfonamide sensitivity, the parent culture was grown in plain beef heart infusion broth for the same time and subcultured at the same intervals as the organisms that were transferred in the sulfapyridine medium. At the end of these transfers, the sensitivity of the control culture was determined in both liquid and solid media containing sulfapyridine.

Results. The steps involved in the conversion of the sensitive strain to a resistant strain have been summarized in figure 1. From these data it is readily apparent that resistance was acquired progressively during the first five serial transfers through media containing sulfapyridine (cf. Exper. 1 to 5, fig. 1). The magnitude of the change in sensitivity is indicated by the fact that growth of the parent organisms (Exper. 1) was inhibited markedly in media containing 5 mgm. % sulfapyridine whereas that of organisms obtained from the fifth serial transfer

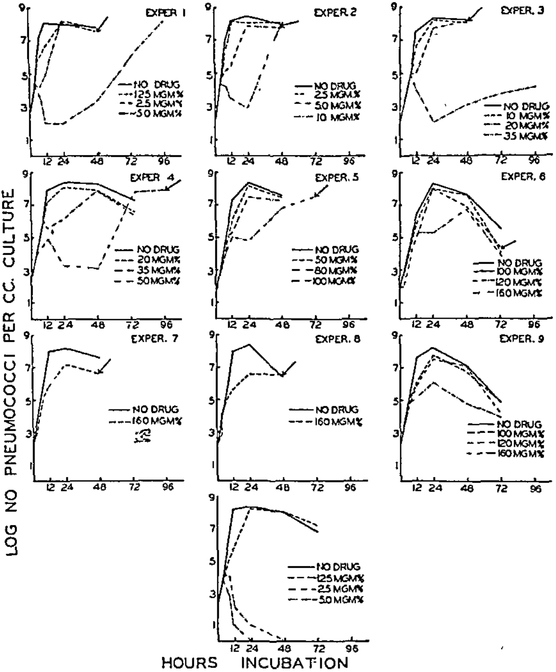

Fig. 1. Development of a Sulfonamide-Resistant Strain of Type II Pneumococcus

Experiment 1. Effect of sulfapyridine on growth of the sensitive parent strain.

Experiment 2. Effect of sulfapyridine on growth of organisms isolated from Exper. 1 (culture containing 2.5 mgm. % sulfapyridine) at time indicated by arrow.

Experiment 3. Effect of sulfapyridine on growth of organisms isolated from Exper. 2 (culture containing 10 mgm. % sulfapyridine) at time indicated by arrow.

Experiment 4. Effect of sulfapyridine on growth of organisms isolated from Exper. 3 (culture containing 20 mgm. % sulfapyridine) at time indicated by arrow.

Experiment 5 Effect of sulfapyridine on growth of organisms isolated from Exper. 4 ... arrow.

... solated from Exper. 5 ... arrow.

... solated from Exper. 6 ... arrow.

... solated from Exper. 7 ... arrow.

... l from Exper. 8

... rain after eight

was essentially as rapid in media containing 100 and 120 mgm. % of the drug as in control media (Exper. 6). After the sixth serial transfer, two additional passages through broth containing 160 mgm. % sulfapyridine had little effect on sulfonamide resistance (cf. Exper. 7, 8 and 9). This finding is of interest, since it may indicate that exposure to successively higher concentrations of sulfonamide is required for development of increased resistance.

The data on the sensitivity of the individual pneumococci in the original culture are shown in table 1. According to these data, only about a third of some 500 organisms were able to grow to a limited degree in media containing 0.6 mgm. % sulfapyridine, and not one of the 500 was able to produce visible growth in agar containing 1.25 mgm. % of this drug. Not one of 50,000 parent pneumococci (twice the number used in the entire inoculum for each vessel in Exper. 1, fig. 1) was able to grow in agar containing 5 mgm. % sulfapyridine. Whether the whole culture contained any organisms that would grow in 5 mgm. % or more

TABLE 1

The sulfapyridine sensitivities of the type II CH pneumococci composing the parent culture

CULTURE DILUTION PLATED	NUMBER OF COLONIES ON PLATE									
	Mgm. per cent sulfapyridine in medium									
	0	0.3	0.6	1.25	2.5	5.0	10	20	40	80
10^{-6}	520	341	163*	0	0	0				
10^{-4}	52,000†					0	0	0	0	0
10^{-3}	520,000†					‡	‡	‡	‡	‡

* Colonies smaller than those on control plate.

† Colony numbers calculated from results on plate of 10^{-6} dilution.

‡ No visible colonies were present, but plate showed same discoloration that characterizes overgrowth. Viable pneumococci were present in the agar since culture of agar cubes from plates yielded positive growth of type II pneumococci.

sulfapyridine could not be determined by this method, since technical difficulties precluded the use of inocula of more than 50,000.

These results are to be compared with the data in table 2, which shows the sensitivities of the type II pneumococci at various stages in the conversion of the sensitive to the resistant strain. It will be noted that upon each additional exposure to sulfapyridine (up to the eighth exposure), organisms were formed which were more resistant to this sulfonamide than the organisms present in the preceding culture. Prior to passage in broth containing sulfapyridine (cf. table 1), the pneumococci were unable to grow in more than 0.6 mgm. % of this drug; after a single passage (cf. table 2), growth was obtained in agar containing 2.5 mgm. %; after the second passage, growth occurred in 5 mgm. %; after the third passage, in 20 mgm. % and after the fourth passage, in 80 mgm. %. All of the cultures that had undergone five, six, seven and eight transfers contained some pneumococci that grew in agar containing 144 mgm. % sulfapyridine, but the proportion of organisms growing in this concentration became larger as

the number of transfers increased. It is noteworthy that in no instance did the colonies grow as well in the agar containing 144 mgm. % sulfapyridine as in the control agar.

Attention should be called to the finding that both the organisms composing the parent culture and those composing cultures of various degrees of resistance were not entirely uniform in their reactions to the sulfonamide. With one exception (Exper. 2), some organisms in each culture were slightly more resistant to the drug than others, as shown by the fact that the plates with the highest sulfapyridine concentrations contained fewer colonies than the plates with the next lower drug concentration.

TABLE 2

Sulfapyridine sensitivities of pneumococci at various stages in the conversion of a sensitive strain to a resistant strain

EXPERIMENT NUMBER (CF. FIG. 1)	NUMBER OF PREVIOUS TRANSFERS IN SULFAPYRIDINE MEDIUM	NUMBER OF COLONIES ON PLATE*										
		Mgm per cent sulfapyridine in medium										
		0	0.3	0.6	1.25	2.5	5	10	20	40	80	144
2	1	136	130	112	114	125‡	0					
3	2	99			131	124	83‡	1§	0			
4	3	296					258	280	128	0	0	
5	4	174						130	96	90	70§	
6	5	127							130	109	74‡	11‡
7	6	269								232	162‡	30‡
8†	7	332								340	316	200‡
9	8	187								205	207	104‡
10	0 (control)	188	192	159‡	0	0						

* The colony counts recorded in this table were obtained from plates prepared from 10^{-6} dilutions of culture Plates of 10^{-5} and 10^{-7} dilutions were also prepared, but the colony counts on these plates are not presented here since they were in agreement with those recorded.

† Colony counts made after 48 hours incubation.

‡ Colonies small in size

§ Colonies minute

Finally, it should be noted that the results of the control experiments (Exper. 10, fig. 1 and Exper. 10, table 2) show that continued passage of the parent culture through beef heart broth was not in itself responsible for the changes in sensitivity described above.

Comment. The data presented in this experiment offer little support for the conception that resistant strains originate through the selection of an occasional resistant organism formed during normal multiplication of the sensitive strain. However, the limitations of the methods used in this study prevent complete elimination of this possibility. It may be that in the normal multiplication of the sensitive strain, extremely few resistant organisms are formed—perhaps fewer than one in one million. It should be pointed out that if resistant organisms were formed so infrequently, resistant strains could not be formed at will from inocula

of 200 to 1000 organisms, as has been done repeatedly in the above experiments and in those described in our previous studies (1).

The results obtained in the current work support the idea that sulfonamide-resistant organisms are formed through some action of these drugs on the sensitive organisms. The data show that upon the initial exposure to the sulfonamide, pneumococci were formed which were more resistant than any of the organisms present in the original inoculum. Repeated exposures to increasing amounts of the drug led to the production of organisms of progressively greater resistance. This stepwise formation of resistant organisms is an indication that the property of sulfonamide resistance is a gradually acquired characteristic. The degree to which this property can be developed is unknown, but it seems reasonable to believe that the use of higher concentrations of the sulfonamide than was possible in our experiments would have enhanced this characteristic.

Little can be said of the manner in which the sulfonamides induce formation of resistant organisms or of the nature of the characteristic responsible for resistance. It has been pointed out that many bacterial variations can be induced by exposure of microorganisms to injurious agents (3), and it has already been suggested that one of the variants produced in response to the action of a sulfonamide may be a resistant organism (1, 4). Whether the characteristic of resistance represents the acquisition or the loss of certain enzymatic activities cannot be stated at present. It has been indicated, however, that certain sensitive strains differ from their corresponding resistant strains in such properties as the production of hydrogen peroxide, the oxidation of glycerol, pyruvate and lactate, and the formation of sulfonamide-inhibiting materials (similar in action to *p*-aminobenzoic acid) (5, 6). It has not been possible to demonstrate, however, that these differences are characteristic of all sensitive and resistant strains (7, 8).

II. A COMPARISON OF THE SULFAPYRIDINE SENSITIVITIES OF PNEUMOCOCCI COMPOSING OTHER SENSITIVE AND RESISTANT STRAINS. In this study the sensitivities of organisms composing resistant strains of pneumococcus were compared with those of organisms composing the parent sensitive strains. This comparison was carried out using the sulfapyridine-agar plate method described above.

METHODS. *Organisms.* The sensitive pneumococci used in this study were the type I McGovern, type III Wistuba and type III CHA strains, all isolated from cases of pneumococcal pneumonia prior to the advent of sulfonamide therapy. The resistant strains which were used had been derived from each of the above sensitive strains by an *in vivo* procedure described before (1). Both sensitive and resistant strains had been passed through mice either daily or on alternate days for two to four years. During this period repeated *in vivo* and *in vitro* tests of sulfonamide sensitivity were carried out, the results of which showed that all six strains were remarkably stable in their reactions to these drugs. The ability of these different strains to grow in sulfapyridine broth was determined just prior to the present experiment (table 3). The methods used in this test have been described elsewhere (9).

Measurement of sensitivity. A culture of the desired strain, prepared from the heart blood of a passage mouse, was subcultured twice in beef heart infusion broth enriched with 2% rabbit blood, each subculture being incubated 12 hours at 37.5°C. The second subcul-

ture was diluted serially in beef heart broth, and 0.5 cc. quantities of a 10^{-5} dilution were plated in various concentrations of sulfapyridine agar in the manner described above. Colony counts and estimations of colony size were made after these plates had been incubated 24 hours at 37.5°C. Three to five different tests of sensitivity were made on each strain, essentially identical results being obtained in these repeated tests.

RESULTS. The results of typical tests with the various organisms have been summarized in table 4. According to the data in this table, each of the resistant strains contained pneumococci that were more resistant to sulfapyridine than any organism found in the corresponding sensitive strain. Thus approximately 5% of the organisms of the resistant McGovern strain produced visible growth in agar containing 40 mgm. % sulfapyridine, and all of the resistant organisms were able to grow in the presence of 20 mgm. % drug; on the other hand, none of the

TABLE 3

Growth of sensitive and resistant strains of pneumococcus in media containing sulfapyridine

ORGANISM	INOCULUM, NUMBER OF PNEUMOCOCCI PER CC. OF CULTURE	VISIBLE GROWTH															
		After 12 hours incubation								After 24 hours incubation							
		Mgm per cent sulfapyridine in medium								Mgm per cent sulfapyridine in medium							
		0	0.6	1.25	2.5	5	10	20	40	0.6	1.25	2.5	5	10	20	40	80
Type I McGovern																	
Sensitive strain ...	1200	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−
Resistant strain.....	1800	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
Type III Wistuba																	
Sensitive strain ..	600	+	+	−	−	−	−	−	−	+	+	+	−	−	−	−	−
Resistant strain....	1000	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
Type III CHA																	
Sensitive strain ..	1400	+	+	−	−	−	−	−	−	+	+	+	−	−	−	−	−
Resistant strain..	1080	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−

pneumococci of the sensitive McGovern strain were able to grow in more than 2.5 mgm. % of the sulfonamide, and only four of each 600 organisms were able to grow in this concentration. Likewise most of the resistant Wistuba organisms were able to grow well in agar containing 20 mgm. % sulfapyridine, whereas only one of each 300 sensitive organisms produced visible growth in a drug concentration of 2.5 mgm. %. The difference in the sulfonamide sensitivities of the sensitive and resistant CHA strains was less striking than that of the McGovern and Wistuba strains. Nevertheless, the resistant CHA strain contained pneumococci that could grow in a sulfapyridine concentration of 20 mgm. %, while none of the pneumococci of the sensitive strain were able to grow in more than the 5 mgm. % concentration.

The data in table 4 also bring out some interesting and important facts concerning the differences in the reactions of individual pneumococci that compose

the various strains. The organisms making up the sensitive McGovern and Wistuba strains were relatively uniform in respect to sulfonamide sensitivity; this was also true of the organisms found in the resistant strains. On the other hand, each CHA strain contained pneumococci which showed significantly different responses to the drug. Two-thirds of the organisms in the sensitive strain were unable to grow in more than 0.6 mgm. % sulfapyridine and only to a limited extent in that concentration. The remaining third of the pneumococci of this strain were able to grow well in agar containing 0.6 and 1.25 mgm. % drug, fairly well in the 2.5 mgm. % concentration and to a limited degree in 5 mgm. % of the

TABLE 4

Comparison of the sulfapyridine sensitivities of pneumococci composing sensitive and resistant strains

ORGANISM	NUMBER OF COLONIES ON PLATE*										
	Mgm. per cent sulfapyridine in medium										
	0	0.3	0.6	1.25	2.5	5	10	20	40	80	144
Type I McGovern											
Sensitive strain...	600	642	608	512	4§	0	0	0	0	0	0
Resistant strain...	900					900	954	1056	42§	0	0
Type III Wistuba											
Sensitive strain...	303	300†	320‡	268§	1§	0	0	0	0	0	0
Resistant strain...	460					402	350	250‡	0	0	0
Type III CHA											
Sensitive strain...	730	840	{110§, 228}	197	110‡	40§	0	0	0	0	0
Resistant strain...	540					610	560†	{88§, 26‡}	0	0	0

* The colonies on plates containing sulfapyridine were comparable in size to those on control plates except when indicated otherwise.

† Indicates that the colonies were slightly smaller than those on control plates.

‡ Indicates that the colonies were small but readily visible.

§ Indicates that the colonies were minute.

sulfonamide. Although the variations among the resistant CHA organisms were not as striking as those of the sensitive strain, the resistant strain did contain organisms of different sensitivities. One group grew fairly well in the presence of 20 mgm. % sulfapyridine whereas the other group produced only minute colonies in that drug concentration.

COMMENT. The finding that the organisms composing the resistant McGovern, Wistuba and CHA strains were more resistant to sulfapyridine than the most resistant pneumococci of the corresponding sensitive strains adds further support to the conception that resistant organisms are produced as a consequence of some action of the sulfonamide on the sensitive organisms.

Although our experiments have failed to demonstrate the existence of any highly resistant organisms in a culture of a sensitive strain, they do show that the organisms of any sensitive strain are not entirely uniform in their reactions to a sulfonamide[1] and that some strains such as the type III CHA show considerable variation in this respect. Since these strains have never been exposed to a sulfonamide, it seems probable that naturally occurring variations in the characteristics of an organism influence its reactivity to these drugs. This finding complicates work on the origin of sulfonamide-resistant strains, for it indicates that breeding-out of the more resistant forms present in a sensitive strain as well as the alteration of these forms through exposure to a sulfonamide may be involved in the production of highly resistant organisms.

In conclusion it might be well to point out that in view of the above finding, any study dealing with the question of the origin of sulfonamide-resistant organisms will have to distinguish between relatively small differences in resistance occurring naturally in a sensitive strain and the high degree of resistance acquired upon exposure of sensitive organisms to a sulfonamide. Recognition of this point is particularly important in clinical studies of sulfonamide resistance where it is desired to know whether changes in sensitivity occur during treatment, since it is not always possible to obtain and study large numbers of organisms from each specimen taken from the patient.

SUMMARY

Experiments were carried out to determine whether sulfonamide-resistant pneumococci are formed during the normal multiplication of a sensitive strain or whether they are produced in response to some action of the sulfonamide on the sensitive organisms. A quantitative study of the sensitivities of individual pneumococci composing a sensitive strain and the changes that occurred during conversion of this strain to a highly resistant strain showed that the pneumococci present after the first exposure to sulfapyridine were significantly more resistant than any organisms in the original sensitive strain. Within certain limits, organisms of increased resistance were formed upon each additional exposure to the drug. A comparison of the sensitivities of individual pneumococci composing other sensitive and resistant strains showed that in every instance the organisms that made up the so-called resistant strains were significantly more resistant than any pneumococci in the sensitive strains. These findings support the conclusion that highly resistant pneumococci are formed as a result of some action of the sulfonamide on the sensitive organisms.

It should be added that the individual pneumococci that make up either a sensitive or a resistant strain are not entirely identical in their reactions to a sulfonamide. These differences in the reactions of individual organisms in any strain are not to be confused with the large differences in sensitivity existing between so-called sensitive and highly resistant strains.

[1] This finding is supported by the observations of Frisch (10) and Horsfall (11).

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DEGRADATION PRODUCTS OF DILANTIN[1]

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Renewed interest in the metabolism of hydantoins has arisen because of the introduction of dilantin (*5,5* diphenylhydantoin) for the treatment of epilepsy. While a considerable amount of work has been reported on the metabolism and chemical behavior of hydantoin and substituted hydantoins, no such work has been done on the *5,5* diphenyl derivative.

To investigate the metabolism of this drug various possibilities were considered. The course of breakdown could follow one or other of two general pathways. One is that the hydantoin ring could be completely disrupted by the splitting off of urea, with the formation of diphenylacetic acid. This deserves consideration since Wada (1) has reported the presence of an enzyme in milk, blood, pancreas and liver extracts, which supposedly splits urea from various hydantoins and hydantoic acids. It would not be likely that the breakdown would proceed further since Miriam, Wolf and Sherwin (2) have shown that diphenylacetic acid when fed to dogs is excreted unchanged to about 75%, while the remainder is eliminated as a conjugated glycuronate.

Another possibility is that there may occur the splitting of the hydantoin nucleus between the 3 and 4 positions with the formation of hydantoic acid, since Gaebler and Keltch (3) have demonstrated that approximately 50% of hydantoin is excreted as hydantoic acid. A possible further breakdown of the hydantoic acid could occur by splitting off of the carbamino group to form the corresponding amino acid. A further breakdown of this amino acid could follow one or other of two well established pathways for the metabolism of amino acids. The first is deamination with the formation of an hydroxyl acid (benzilic acid) or an unsubstituted acid (diphenylacetic acid). Sieburg and Harloff (4) have demonstrated that benzilic acid is excreted unchanged by rabbits, which is to be expected since Miriam *et al.* pointed out that the strong acidic character of this substance would be likely to prevent its metabolism. The presence of a keto acid is obviously impossible since three of the four valences of the alpha carbon atom are already satisfied; consequently an oxidative deamination of the amino acid could not occur.

Another reaction which must be considered is decarboxilation of the amino acid with the formation of an amine. Amines are usually quite unstable and detoxification of amines takes place by removal of the amino group with the formation of an alcohol, ketone or hydrocarbon. These products may be excreted as such or oxidized still further. Still another reaction for the breakdown of the amino acid should be considered. It appeared conceivable that the

[1] Supported by grants from the Wisconsin Alumni Research Foundation. Dilantin was supplied by Parke, Davis & Company.

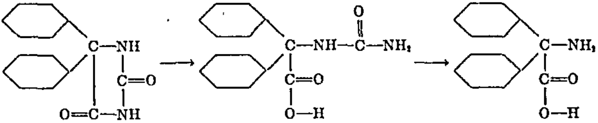
phenyl groups may offer a point for attack in that one or both phenyl groups may be removed from the alpha carbon atom and be excreted as phenols or benzoic acid.

Qualitative examination of urine. Urine from dogs which received dilantin intravenously was examined for the presence of unchanged drug, diphenyl acetic, diphenyl hydantoic, benzilic, and alpha amino diphenyl acetic acids, diphenyl amino methane, benzophenone, and an increase in phenol bodies. By means of the colorimetric technique described by Folin and Denis (5) no increase in phenol bodies could be demonstrated, nor was there any increase in the percentage of organic sulfates. These results indicate that the phenyl groups are not excreted as phenol bodies.

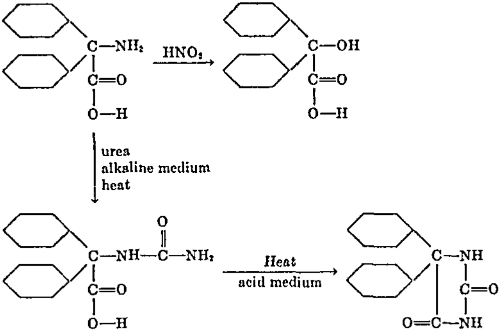
Diphenyl acetic and benzilic acids, diphenyl amino methane, and benzophenone can be quantitatively recovered from urine by extraction with ether in a continuous extractor and subsequent sublimation under reduced pressure. When small quantities of these compounds were added to urine specimens, recoveries of 95% or higher were obtainable with this technique. Likewise when benzilic and diphenyl acetic acids were administered to dogs, they were easily demonstrable in the urine. On the other hand, benzophenone and diphenyl amino methane apparently are destroyed since they could not be found in the urine after their administration. None of these compounds could be demonstrated in the urine after the administration of dilantin.

To examine urine for the presence of diphenyl hydantoic and alpha amino diphenyl acetic acids, the urine was concentrated on a steam plate and extracted under reduced pressure with butyl alcohol in a continuous extractor. The extracted residue was then washed with successive portions of hot 95% ethyl alcohol in order to remove the urea, phenols, and urinary pigments contained in the residue. The acids were partially purified by recrystallizing them from dilute ammonium hydroxide. The product was further purified by sublimation under reduced pressure. The melting point of the purified product was 242°C., as compared with the melting point of alpha amino diphenyl acetic acid of 248°C., reported by Biltz and Seydel (6). A mixed melting point was not determined because the pure compound was not available to us; however, its identity was satisfactorily established by converting it to benzilic acid, which was subsequently identified by its melting point and mixed melting point. Attempts at isolation of diphenyl hydantoic acid were unsuccessful. However, since this acid must be considered a precursor of alpha amino diphenyl acetic acid, its presence in at least small quantities must be assumed. Its presence was established indirectly. Following acid treatment of the urine from dogs receiving dilantin, there was always an increase in the amount of diphenylhydantoin over that found in untreated urine. Since the closing of the ring by acid treatment is a reaction characteristic of the hydantoic acids, the presence of diphenyl hydantoic acid is indicated.

The formulas for the degradation products and the pathway of metabolism are given below:



Reactions employed in identifying the degradation products are:



QUANTITATIVE RECOVERIES OF THE DEGRADATION PRODUCTS. In order to determine the relative amounts of unchanged hydantoin, hydantoic and alpha amino diphenyl acetic acids which are excreted in the urine, the technique previously described for the quantitative determination of the hydantoin was employed (7). The conversion of amino acids into the corresponding hydantoic acid and then into the hydantoin are well established reactions and require no detailed discussion. The first step of the conversion takes place in a slightly alkaline medium, the amino acid being refluxed with either an excess of urea or ammonium cyanate to form the hydantoic acid. Subsequent heating in an acid medium closes the ring to form the corresponding hydantoin. After dogs or patients had received known amounts of the drug, the urine was collected throughout a period of forty-eight hours after the last administration. Each specimen was divided into three equal aliquots. One aliquot was extracted directly with ether in a continuous extractor. Since only the unchanged drug is soluble in ether, the degradation products are not extracted. The second aliquot was acidified and heated on a hot plate for twenty-four hours and then extracted with ether. The quantity of drug recovered in this fraction represents the unchanged hydantoin plus the drug reconstituted from the hydantoic acid. The third aliquot was first reduced in volume and then refluxed with an excess of urea and subsequently acidified and heated on a hot plate. The recovered

hydantoin in this fraction represents the unchanged drug plus the drug reconstituted from the hydantoic and amino acids. The relative amounts of each of these compounds were calculated by employing the appropriate gravimetric factors. The results which were obtained on man and dog are given in table 1.

DISCUSSION. It is apparent from the results obtained in this investigation that the mechanism of degradation of *5,5* diphenylhydantoin follows that of hydantoin. In both cases the preliminary step in the destruction of the nucleus is a rupture of the ring between the *3* and *4* positions. However, the destruction of *5,5* diphenylhydantoin proceeds further than when the *5* position is unsubstituted, since the corresponding amino acid actually appears in the urine. The two phenyl groups render the nucleus less stable since, according to Gaebler and Keltch, after the administration of hydantoin, 50% is excreted unchanged and the remainder as the hydantoic acid. Only 1 to 4% of the *5,5* diphenylhydantoin is excreted unchanged and only about 35% of the administered drug can be accounted for either in the unchanged form or its two degradation products. The remaining 65% apparently is completely destroyed by a further breakdown of the amino acid.

TABLE 1

SUBJECT	DOSE	ROUTE OF ADMINISTRATION	PERCENTAGE EXCRETED AS		
			Unchanged	Corresponding hydantoic acid	Alpha amino diphenylacetic acid
	grams				
Man	0.5	Oral	0.6	Not determined	10.3
Man	0.5	Oral	0.3	1.2	13.6
Man	0.5	Oral	0.7	1.1	19.3
Man	0.5	Oral	1.2	0.2	28.6
Dog	1.0	Intravenous	4.0	Not determined	13.4
Dog	1.5	Oral	0.6	Not determined	20.0
Dog	2.5	Intravenous	4.6	Not determined	27.2
Dog	2.0	Intravenous	4.0	4.6	27.2
Dog	2.0	Intravenous	2.4	3.0	17.3
Dog	2.0	Intravenous	3.1	3.4	24.2

These results do not substantiate the presence of the enzyme, reported by Wada, because the presence of diphenylacetic acid could not be demonstrated.

SUMMARY

The metabolism of *5,5* diphenylhydantoin (dilantin) was investigated. It was found that in man and dog approximately 1 to 4% of the drug is excreted unchanged, 1 to 5% as the hydantoic acid, and 10 to 27% as alpha amino diphenyl acetic acid. Only 30 to 35% of the administered drug could be accounted for.

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hydantoin in this fraction represents the unchanged drug plus the drug reconstituted from the hydantoic and amino acids. The relative amounts of each of these compounds were calculated by employing the appropriate gravimetric factors. The results which were obtained on man and dog are given in table 1.

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	grams				
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Man	0.5	Oral	0.3	1.2	13.6
Man	0.5	Oral	0.7	1.1	19.3
Man	0.5	Oral	1.2	0.2	28.6
Dog	1.0	Intravenous	4.0	Not determined	13.4
Dog	1.5	Oral	0.6	Not determined	20.0
Dog	2.5	Intravenous	4.6	Not determined	27.2
Dog	2.0	Intravenous	4.0	4.6	27.2
Dog	2.0	Intravenous	2.4	3.0	17.3
Dog	2.0	Intravenous	3.1	3.4	24.2

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The metabolism of *5,5* diphenylhydantoin (dilantin) was investigated. It was found that in man and dog approximately 1 to 4% of the drug is excreted unchanged, 1 to 5% as the hydantoic acid, and 10 to 27% as alpha amino diphenyl acetic acid. Only 30 to 35% of the administered drug could be accounted for.

RESULTS AND DISCUSSION. The results of the metabolism studies are summarized in table 2. Each value represents an average of four or five determinations. The average for the dilantin values is included to serve as a basis for comparison.

TABLE 1

Recoveries of some hydantoin derivatives related to 5,5 diphenylhydantoin

COMPOUND	FACTOR*	MGM ADDED	MGM. RECOVERED	% RECOVERED
Chlorination				
1-acetyl *5,5* diphenylhydantoin	7.35	25.0	24.0	96.0
		50.0	49.5	99.0
		125.0	123.4	98.6
2-thio *5,5* diphenylhydantoin	6.70	50.0	48.9	97.8
		90.0	89.3	99.2
		100.0	100.3	100.3
5-ethyl *5*-phenyl hydantoin	5.10	20.0	19.7	98.5
		35.0	34 8	92.4
		225.0	217.4	96.6
p,p'-dimethoxy *5,5* diphenylhydantoin	7.80	25.0	24.6	98.4
		50.0	48.8	97.6
		100.0	99.2	99.2
Diphenyl hydantoic acid	6 75	40.0	38.0	96.6
		50.0	47.0	94.0
		100.0	93.2	93.2
Alpha amino diphenyl acetic acid	5.67	25.0	23.2	92.8
		50.0	47.4	94.8
		90.0	86.6	96.2
Sublimation				
2-dihydro *5,5* diphenylhydantoin		100.0	99.3	99.3
		125.0	130.5	104.4
		250.0	245.0	98.0
3-methyl *5,5* diphenylhydantoin		35.0	35.7	102.0
		45.0	44.9	99.8
		100.0	97.2	97.2

* Milligrams of drug equivalent to 1 cc. N/10 solution of $Na_2S_2O_3$.

The amount of alpha amino diphenyl acetic acid which appears in the urine after the administration of diphenyl hydantoic or alpha amino diphenyl acetic acid is nearly the same as that found after the administration of equivalent amounts of the parent compound. These results indicate a lack of stability of these two acids *in vivo*, since it would be expected that larger quantities would

appear in the urine after their administration if they represented the end products of hydantoin metabolism. Furthermore, they appear to substantiate the hypothesis that the only means of breakdown of the *5,5* disubstituted hydantoins is by cleavage of the ring between the *3* and *4* positions. The close approximation of the amounts of amino acid excreted after the administration of the hydantoins indicates that they all pass through the amino acid stage enroute to complete destruction. The administration of the hydantoic and amino acids results in a slightly higher percentage of excretion of these acids than when the corresponding hydantoin is administered, apparently due to a higher initial concentration in the blood. This is to be expected since the amount of hydantoic and amino acids in the blood at any time resulting from the metabolism of the hydantoin is undoubtedly quite small.

It will be noted that the excretion of the *1*-acetyl and *2*-thio derivatives is approximately the same as that of the parent compound, *5,5* diphenylhydantoin.

TABLE 2

Excretion of hydantoin derivatives

COMPOUND	PERCENTAGE EXCRETED		
	Unchanged	Hydantoic acid	Corresponding amino acid
Dilantin	3.2	3.9	22.9
Diphenyl hydantoic acid		11.5	19.9
Alpha amino diphenyl acetic acid			27.3
1-acetyl *5,5* diphenylhydantoin	4.1	5.8	20.1
2-thio *5,5* diphenylhydantoin	4.5	6.6	25.1
2-dihydro *5,5* diphenylhydantoin	0.0	0.0	25.7
3-methyl *5,5* diphenylhydantoin	3.3*	2.0	13.3
p,p'-dimethoxy *5,5* diphenylhydantoin	2.2	1.2	4.4
5-ethyl *5*-phenyl hydantoin	15.0	1.1	30.1

* Found as the demethylated compound.

Apparently these groups have no effect on the cleavage of the ring. It must be pointed out in this connection that it is uncertain whether or not deacetylation takes place. The titrimetric values for the unchanged hydantoin and hydantoic acid following hydrolysis with sodium hydroxide are slightly higher than the values before hydrolysis, indicating that a small amount of the acetylated compound may be excreted. It is uncertain whether or not the amino acid remains acetylated since deacetylation occurs *in vitro* during the condensation with urea in dilute alkali. Apparently the hydantoin and hydantoic acids are present as the oxygen analogues following the administration of the thio derivative, since there is no increase in the recoveries following the action of nitric acid on the residues extracted with ether.

The hydrogens in the *2* position of the dihydro derivative do not alter the mechanism of the breakdown since the amino acid is excreted approximately in the same amount as that found after the administration of dilantin. It

appears, however, that the dihydro derivative is broken down more readily than the oxy analogue since neither the unchanged compound nor the corresponding hydantoic acid is excreted. If the hydrogens were removed before the ring was opened it should be possible to demonstrate the presence of the oxygen-containing hydantoic acid; hence the opening of the ring must have preceded the oxidative removal of the hydrogens.

The methyl group in the *3* position renders the hydantoin less reactive *in vitro* but not *in vivo*. Apparently demethylation precedes cleavage of the ring since small amounts of *5,5* diphenylhydantoin and the hydantoic acid appear in the urine, as demonstrable by titration. No methylated compound could be isolated by sublimation. The smaller quantity of the amino acid found is probably due to incomplete absorption from the gastro-intestinal tract.

Modification of the groups in the *5* position significantly alters the extent but does not change the process of breakdown of the drug. Methoxy groups in the para positions of the benzene rings render the compound relatively unstable since this hydantoin and its degradation products can be recovered to approximately one-third of the amount obtained from dilantin. Conversely, the presence of an ethyl group in the *5* position stabilizes the ring, 15% of the drug being excreted unchanged compared with 3% of the administered dilantin Furthermore, the excretion of the amino acid from this ethyl derivative is higher than from any of the other hydantoins.

SUMMARY

Excretion studies on the degradation products, which so far have been identified as coming from dilantin and hydantoin derivatives closely related to dilantin, are presented. The acetyl group in the *1* position, the sulfur in the *2* position, and the methyl group in the *3* position have no effect on the stability of the hydantoin molecule *in vivo*. On the other hand, methoxy groups in the para positions of the benzene rings of dilantin appear to render the molecule less stable, while an ethyl group in the *5* position in place of one of the phenyl groups renders the molecule more stable.

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THE ANALGESIC PROPERTIES OF CERTAIN DRUGS AND DRUG COMBINATIONS

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In a recent paper (1) we described a new and simple method for assaying the analgesic potency of drugs. The simplicity of the method recommended its use for a more detailed study of certain phases of the analgesia problem, with particular reference to the possibilities of inducing analgesia with combinations of the non-opiate drugs and of potentiating the analgesic action of the opiates with non-opiate drugs.

EXPERIMENTAL. The apparatus used was the same as that previously described except that storage batteries were used as the source of current, to avoid fluctuations in line voltage. It is an adaptation of the method reported by Hardy and Wolff (2) for determination of the pain threshold in humans, i.e. a beam of light of constant intensity is focused on the tip of a rat's tail and when pain is felt the tail is quickly flipped aside. The reaction time is remarkably constant. Administration of an analgesic delays the response to this pain sensation, or, in appropriate doses, abolishes it altogether. A severe burn may be inflicted without any response although the animal is completely conscious. It is in this sense that the term "analgesia" is employed in this paper.

System of Scoring. In the previous paper complete loss of the sensation of pain was used as the criterion of analgesia. However, since with low dosages complete analgesia may not be produced, a method of scoring was adopted which would take into account slighter degrees of analgesia. Since thousands of tests have demonstrated the constancy of response in the untreated rat, it seemed justifiable to consider any significant lengthening of response as due to the action of the analgesic. Throughout this study a constant light intensity was used, of such strength that the average response occurred within five seconds. We arbitrarily assigned the following values to lengthening of the reaction time: 7.0 to 7.5 seconds = 1; 8.0 to 8.5 seconds = 3; 9.0 to 9.5 = 6; 10 seconds or more = 10. Table 1 will illustrate the scoring schedule used.

Since all results in this study are expressed in terms of "Degree Analgesia' an explanation of table 1, which represents an example of the method used in calculating all results, may be desirable. The degree of analgesia is scored in points which, although arbitrarily assigned, express more justly the degree of loss of pain sensation than would be indicated merely from the increase in reaction time. As shown in the previous study (1) very occasionally (3 times in 1,000) an untreated rat will take 6.5 seconds before responding to a constant light intensity having an average effect within 5 seconds. A reaction time of 7.0 to 7.5 seconds therefore means little, and is given only 1 point. On the other hand, a rat which does not respond by the end of 10 seconds will usually not respond at all and is, in other words, completely analgesic. Exposing the tail for longer than 10 seconds, however, results in a bad burn, which is undesirable if the animals are to be used frequently; therefore, 10 seconds was the maximum time of exposure used. This degree of analgesia is obviously much greater than would be expressed merely by an increase from 7.0 to 10.0 seconds. To this degree of analgesia 10 points, the maximum, was therefore assigned. Intermediate values, 3 points for 8.0 to 8.5 seconds and 6 points for 9.0 to 9.5 seconds appear fair, in view of the above.

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Examination of the table shows that rat No. 1 had a maximum reaction time of 9 0 seconds. It receives 6 points. The next rat had not responded at the end of 10 seconds, therefore receives 10 points. The fourth rat did not show even the minimum increase (7.0 seconds) and receives nothing. The points are then totalled. This total is termed the "Degree Analgesia" for the group, in this case, 49. Had no rats in the group had their reaction time increased to 7.0 seconds, the "Degree Analgesia" would have been 0; had all rats failed to respond at the end of 10 seconds, the "Degree Analgesia" would have been 100. In those cases where the group consisted of 12 animals the total score is multiplied by 10/12 to give the "Degree Analgesia" for the group. As stated, this term is used in expressing all of the following results, and wherever it is encountered it means the degree of analgesia demonstrated by a group of rats, calculated as explained in the above paragraphs.

Reliability of Assay Method. It is generally recognized that the factor of individual variation is important in all types of biologic assays. Although the response of untreated rats to pain, as measured by this method, is highly uniform, individual variation to the action of analgesics is great. There is every

TABLE 1

Male rats, dose 4 mgm./kgm. Morphine sulfate

RAT NO.	CONTROL TIME	AFTER 30 MIN.	AFTER 1 HR.	AFTER 2 HRS.	POINTS PER RAT
1	4.5	7.0	9.0	6.0	6
2	5.0	10.0	8 0	5.0	10
3	5.0	8 5	6 0	5.0	3
4	4.5	6.0	5.0	5.0	0
5	5.0	10.0	8.5	6.0	10
6	5.0	8.0	8 0	5 0	3
7	4.0	8.0	9.0	6.0	6
8	4.5	7.5	6.0	4.5	1
9	5.5	9.0	10.0	6.0	10
10	5.0	6 5	5.0	5.0	0
Total					49

"Degree Analgesia" for the group = 49.

reason to think that this variation holds for other species of animals; the simplicity of the method and the use of such a convenient laboratory animal as the rat permits use of the large number of animals necessary for reliability of assay results. The following data are presented to indicate the degree of reliability of the method.

1. Variation between individuals. Response of individual animals to a given dose (4 mgm. per kgm. of morphine sulfate) is shown in table 1. It will be noted that the individual variation is great, some animals showing no analgesic effect, others the maximum degree. Tests on individual animals are therefore of little value and the unit employed is the group, consisting of 10 or 12 animals. Several groups were used to assay those combinations which seemed promising.

2. Variation between groups. Next it is desirable to know how variable the response to a given preparation, from group to group, would be. This information is given in table 2.

Examination of these data shows that, while the results from group to group are variable, they are not out of line with results ordinarily encountered in bio-assay work. Considering the assay of 2 mgm./kgm. of morphine sulfate, where 23 groups of 10 rats each are compared, it is found that the minimum "Degree of Analgesia" in any group was 1, and the maximum 34. (The "Degree of Analgesia" for each group was determined as described in table 1). The mean was 13.5 and the standard deviation 9.48. This indicates that 95 out of each 100 groups chosen at random would fall between 0 and 32.5, with practical certainty that no group would exceed a degree of analgesia of 37.5.

TABLE 2

TYPE RATS	TREATMENT	RATS PER GROUP	NO. GROUPS	DEGREE ANALGESIA			ST.D.	ST.E.
				Min.	Max.	Mean		
	doses in mgm./kgm.							
Males	Morph. sulf. 2, intra-perit.	10	23	1	34	13.5	9.48	2.02
Males	Morph. sulf. 4, intra-perit.	10	5	40	59	50.2	3.2	1.6
Males	Codeine 10, oral	10	7	0	8	3.3	1.03	0.43
Males	Codeine 20, oral	10	10	8	32	21.8	8.45	2.51
Cast. fem.	Dilaudid 1, intra-perit.	12	10	13	38	25.5	10.24	3.4
Cast. fem.	Morph. sulf. 8, intra-perit.	12	6	36	58	49.7	3.23	1.45

TABLE 3

GROUP	TREATMENT	DATE AND DEGREE ANALG.	DATE AND DEGREE ANALG.	DATE AND DEGREE ANALG.	DATE AND DEGREE ANALG.	ST.D.	ST.E.
	doses in mgm./kgm.						
1 c	Dilaud 1	7/15–23	7/31–31	8/28–21		4.3	3.1
2 c	Morph. 8	7/ 8–40	7/23–37	8/18–43	8/28–33	3.7	2.2
3 c	Morph. 8	7/29–49	8/15–44	8/29–40	9/11–32	6.6	3.3
4 m	Morph. 2	1/ 5–33	1/12–24	1/22–19		5.7	4.1
5 m	Morph. 4	1/ 4–44	1/21–55	2/ 1–42		6.0	4.3
6 m	Morph. 2	6/16–17	8/25– 1				
7 m	Morph. 2	7/ 5–12	8/27– 5				
8 m	Codeine 20 (oral)	7/30–22	8/15–15				

Groups 1–7 injected intra-peritoneally. c = castrate females; m = males.

3. Change in sensitivity with time. The data obtained in table 2 are taken from random groups of rats. It was next desired to know whether a given group, used repeatedly, would give a more uniform response than random groups and also whether their sensitivity would increase or decrease with time. Table 3 shows the response of the same groups of animals to repeated doses of opiates, over a period of time.

Examination of table 3 will show that there is a somewhat greater uniformity in results if the same group of animals is used at intervals than when random groups are used, as indicated in the generally smaller standard deviations. It is also apparent that there is a progressive loss of sensitivity with continued use.

(This difference is statistically significant, the critical ratio being 4.3, indicating practical certainty that the change is not due to chance.) These findings apply to the later work reported in this paper, in which attempts were made to potentiate the action of opiates. In those studies the same animals were used, first with the opiate alone, and later with the opiate plus the potentiating agent. This method takes advantage of the greater uniformity in response shown above. Also, any potentiation observed would actually be greater than indicated, since some refractoriness would have been overcome as well.

4. Strain and sex variations. Only two strains were available for comparison, the University of Denver and the Stanford University strains. As to sex, in our experience the female is less suitable than the male as the latter is tamed more easily and tends to lie quietly while his tail is being burned. However, we had available a colony of castrate females which had been handled a good deal. Table 4 gives a comparison of the different types of rats used.

It appears that the University of Denver rats, which were used exclusively in the further studies, are somewhat more sensitive than Stanford University rats and that males, also used exclusively in subsequent studies, are also more sensitive.

TABLE 4

TYPE	NUMBER	TREATMENT	DEGREE ANALGESIA
Stanford males	30	2 mgm./kgm. morph.	6.0
U. of Denver males	100	2 mgm./kgm. morph.	11.4
U. of Denver males	50	4 mgm./kgm. morph.	50.1
U. of Denver cast. females	50	4 mgm./kgm. morph.	21.4

The foregoing paragraphs have dealt with assay details. Although variations between individual rats are great and even when used in reasonably large groups considerable variations still exist, the method, in view of its convenience, seemed satisfactory to use in a study of the possible analgesic action of certain drug combinations and in an effort to potentiate the action of opiates. These results will now be given.

1. Attempts to obtain analgesic effects with non-opiate drugs and drug combinations. A number of drugs, aspirin, aminopyrine, antipyrine, etc., obtund pain and are classified as analgesics. The barbiturates, although not rated as analgesics are central nervous system depressants. It is not known with certainty exactly how any of these drugs exercise their effects. The possibility therefore exists that certain combinations or dosages of such drugs might be found which would act synergistically to give a degree of analgesia comparable to that of the more potent opiates. The results are presented in three tables: table 5 includes combinations of the so-called analgesics, table 6 the barbiturates alone and in combination with the analgesics and table 7 a study of miscellaneous substances. Each group includes 10 to 20 animals.

The studies reported in the foregoing tables were intended only to bring to

TABLE 5

Subcutaneous			Oral		
Drug	Dose	Degree analgesia	Drug	Dose	Degree analgesia
	mgm./kgm.			*mgm./kgm.*	
Aspirin	400	2	Aspirin	450	33
Aspirin	600	0			
Aminopyrine	150	11	Aminopyrine	450	31
Aminopyrine	300	64	Aminopyrine	600	28
Quinine sulfate	300	0	Quinine sulfate	600	0
Antipyrine	300	2	Antipyrine	900	6
Antipyrine	600	39			
Acetanilid	600	20	Acetanilid	800	13
Acetophenetidin	600	0	Acetophenetidin	700	15
			Acetophenetidin	900	24
Na salicylate	300	0	Na salicylate	520	8
Aminopyrine	150	32	Aminopyrine	300	0
Aspirin	200		Aspirin	400	
Aminopyrine	150	58	Aminopyrine	150	0
Antipyrine	300		Antipyrine	300	
Aminopyrine	100		Aminopyrine	150	
Antipyrine	200	23	Antipyrine	200	6
Aspirin	100		Aspirin	100	
Aminopyrine	150	29	Aminopyrine	225	5
Acetanilid	300		Acetanilid	450	
Aminopyrine	150	31	Aminopyrine	300	0
Na salicylate	300		Na salicylate	500	
Aminopyrine	150		Aminopyrine	225	
Antipyrine	200	59	Antipyrine	300	46
Na salicylate	300		Na salicylate	450	
Aspirin	300	39	Aspirin	500	0
Acetophenetidin	300		Acetophenetidin	450	
Aspirin	200	5	Na salicylate	300	11
Antipyrine	300		Antipyrine	300	
Acetanilid	300	0	Na salicylate	500	2
Acetophenitidin	300		Aspirin	500	
			Antipyrine	450	
			Acetanilid	450	12
			Aminopyrine	225	
			Acetanilid	225	
			Aminopyrine	225	4
			Acetophenetidin	225	
			Quinine sulfate	450	10
			Aminopyrine	225	

TABLE 6

GROUP	MATERIAL	DOSE	ROUTE	DEGREE ANALGESIA
		mgm./kgm.		
1	Cyclopal	90	Sub-cut.	7
2	Cyclopal	90	Oral	0
3	Pentobarbital Na	30	Sub-cut.	12
4	Allonal*	60	Sub-cut.	1
5	Barbital	45	Sub-cut.	0
6	Veranol	45	Sub-cut.	0
7	Ortal Na	60	Sub-cut.	0
8	Mebaral	60	Sub-cut.	0
9	Phenobarbital	20	Sub-cut.	0
10	Seconal	30	Sub-cut.	0
11	Delvinal Na	25	Sub-cut.	0
12	Cibalgin†	200	Oral	22
13	Evicyl‡	250	Oral	10
14	Cyclopal Aminopyrine	45 150	Sub-cut.	24
15	Cyclopal Aminopyrine	45 150	Oral	0
16	Cyclopal Antipyrine	50 600	Sub-cut.	10
17	Cyclopal Acetyl sal. Aminopyrine	50 200 150	Sub-cut.	30
18	Cyclopal Aminopyrine Antipyrine	50 150 300	Sub-cut.	30
19	Cyclopal Aminopyrine Acetanilid	50 150 300	Sub-cut.	21
20	Cyclopal Aminopyrine Na salicylate	50 150 200	Sub-cut.	54
21	Cyclopal Acetophenitidin Acetyl sal.	50 200 200	Sub-cut.	1
22	Cyclopal Aminopyrine Antipyrine Na salicylate	25 100 150 100	Sub-cut.	37

* 60 mgm. Allonal consists of 15 mgm. Allyl-isopropyl-barbituric acid and 45 mgm. acetophenitidin.

† 200 mgm. Cibalgin consists of 25 mgm. diallyl-barbituric acid and 175 mgm. aminopyrine

‡ 250 mgm. Evicyl consists of 30 mgm. *N*-methyl-cyclohexenyl-methyl-barbituric acid and 220 mgm. acetyl salicylic acid.

light outstanding analgesic effects, if such occurred, in these groups of drugs. The number of animals used was too small (10 to 20 in each group) for adequate statistical analysis. It will be noted that little analgesia was produced by the non-opiate drugs, in the combinations studied, unless the dose was very large. The best appear to be aminopyrine, sodium salicylate and antipyrine.

2. Attempts to potentiate the analgesic action of opiates. We next turned our attention to the possibility of potentiating the action of opiates by means of non-opiate drugs. It was felt that if some combination were found whereby

TABLE 7

GROUP	MATERIAL	DOSE	ROUTE	DEGREE ANALGESIA
		mgm./kgm.		
1	Seconal Stilbestrol*	30 .2	Sub-cut.	0
2	Seconal Testosterone prop.*	30 .2	Sub-cut.	0
3	Dilantin Na	50	Oral	0
4	Cyclopal Dilantin Na	25 50	Oral	0
5	Seconal Cyclopal Chloral hydrate	12.5 12.5 20	Oral	0
6	Seconal Cyclopal Chloral hydrate	12.5 12.5 12.5	Sub-cut.	0
7	Pentobarbital Atropin sulf.	20 2	Oral	0
8	Pentobarbital Atropin sulf.	20 2	Sub-cut.	0
9	Scopolamine	1.5	Sub-cut.	0
10	Delvinal Na Scopolamine	25 1.5	Sub-cut.	0
11	Delvinal Na Scopolamine Dilantin Na	25 1.5 50	Oral	0

* Given 24 hours previously.

the dose of the opiate could be significantly reduced such a finding would have value, (perhaps especially in war medicine) in reducing the danger of addiction and in conserving available supplies. The results of these studies are presented in table 8. A few miscellaneous agents, prostigmine, quinine, scopolamine and two steroid hormones, stilbestrol and desoxycorticosterone acetate, were also investigated, because of suggestions in the literature. These results are shown in table 9. It should be noted that the same rats are used in each potentiation experiment. Thus, in table 8, group 9, the same 40 rats whose degree of anal-

TABLE 8
All doses in mgm./kgm.

GROUP	NO RATS	ROUTE	OPIATE	POTENTIATING AGENT	DEGREE ANALGESIA
1	40	I.P.	None	Cyclopal, 25, aminopyrine 40	8.4
2	20	I.P.	Morph. 2	None Aminopyrine 40	19.5 35.7
3	30	I.P.	Morph. 2	None Cyclopal, 25	8.3 17.5
4	50	I.P.	Morph. 2	None Aminopyrine 40, cyclopal 25	16.2 82.8*
5	50	I.P.	Morph. 2	None Na salic. 100, cyclopal 25	12.6 58.2*
6	20	I.P.	Codeine 10	None Aminopyrine 40	17.0 65.0*
7	20	I.P.	Codeine 10	None Cyclopal 25	10.0 68.0*
8	20	I.P.	Codeine 10	None Aminopyrine 20, cyclopal 12.5	20.0 80.0*
9	40	I.P.	Codeine 10	None Aminopyrine 40, cyclopal 25	18.8 91.4*
10	20	I P.	Codeine 10	None Na salic. 100, cyclopal 25	12.5 40.5*
11	10	Oral	None	Aminopyrine 150, cyclopal 45	0
12	30	Oral	Codeine 10	None Aminopyrine 40, cyclopal 25	3.0 13.0
13	30	Oral	Codeine 20	None Aminopyrine 40, cyclopal 25	29.3 53.3
14	40	Oral	Codeine 20	None *Aminopyrine 80, cyclopal 50*	17.5 *49.2**
15	60	Oral	Codeine 120	None	42.6
16	20	I.P.	None	$MgSO_4$ 200, cyclopal 25	25.0
17	20	I.P.	Codeine 10	None $MgSO_4$ 200	5.5 21.0
18	20	I P.	Codeine 10	None Cyclopal 25	17.0 50.5*
19	20	I P.	Codeine 10	None $MgSO_4$ 200, cyclopal 25	18.5 87.5*

I.P. = intra-peritoneally.
All results with asterisk are statistically significant, having a critical ratio of 4 or above.

gesia was 18.8 on 10 mgm. of codeine alone were increased to 91.4 when 40 mgm. aminopyrine and 25 mgm. cyclopal were given in addition. The interval between the control and the potentiation injection was usually two weeks.

The data embodied in table 8 indicate that a significant increase in the analgesic power of both morphine and codeine may be achieved with simultaneous administration of both aminopyrine and cyclopal. This effect is greater if the drugs are given intraperitoneally than orally; however, a significant increase was achieved orally with codeine by doubling the dose of the non-opiate drugs. In general, it appears that the analgesic action of codeine is more easily increased than is the case with morphine, cyclopal alone being effective, and with magnesium sulfate in addition, a very high degree of analgesia was obtained. If substantiated, the findings in respect to codeine may be important, since although it is unquestionably an addictive drug, the impression seems to prevail that it is much less so than morphine.

TABLE 9

All doses in mgm./kgm.

GROUP	NO. RATS	TYPE	OPIATE	POTENTIATING AGENT	DEGREE ANALGESIA
1	20	C. females	Morph. 8	None	55.0
				Prostigmine Br. 0.1	60.5
2	20	C. females	Dilaudid 1	None	33.5
				Prostigmine M.S. 0.2	37.5
3	30	Males	Morph. 2	None	13.6
				Quinine bisulfate 100	7.0
4	10	Males	Morph. 2	None	21.0
				Scopolamine 4	0
5	10	Males	Morph. 2	None	13.0
				Scopolamine 4, cyclopal 25	7.0
6	20	C. females	Morph. 8	None	61.1
				12 hours after desoxycorticosterone acetate 25	53.8
				24 hours after D.C.A. 25	59.0
7	20	C. females	Dilaudid 1	None	29.2
				After 5 days, 0.5 mgm. stilbestrol per day	24.0

In table 9 it is interesting to note that prostigmine, in the doses used, was ineffective in increasing the analgesic action of morphine. Such an effect has been both asserted and denied in the literature. Also interesting is the apparent action of scopolamine and of quinine bisulfate in tending to abolish or reduce analgesic effects. The number of animals used in these latter experiments is, however, too small to be certain of the findings.

Discussion. As stated earlier, the term "analgesia" is used in this paper simply to mean the apparent loss of pain sensation, as indicated by the slowing or loss of response shown by the rat when his tail is burned. Whether an agent producing analgesia of this sort would also be effective in allaying deep-seated pain in the human is not known. There are reasons for believing it would. First, in a previous paper (1) we showed that a comparative assays of five opiates by this method gave results in agreement with their relative values in human therapeutics, as stated in pharmacologic treatises, presumably in allaying pain,

usually, of the deep-seated type. Also, in general, the best potentiation reported in the present study was achieved with agents known to have some degree at least of analgesic properties in the human, such agents being aminopyrine, sodium salicylate and magnesium sulfate. These drugs also exhibit analgesic values as tested by the rat tail method, although the doses must be high.

It will be apparent that the number of drug combinations which have been studied is small, as compared to the almost endless number that might well have been investigated. It is also a question whether the particular drugs and combinations used represent the best choices that could have been made. The effectiveness of aminopyrine as a potentiating agent is unfortunate in view of the possible danger (neutropenia) connected with its use; one wonders to what extent the dosage could be reduced by substituting sodium salicylate or magnesium sulfate, both quite effective. Also, since barbiturates differ chemically, one other than cyclopal, to which most attention was devoted, might be even more effective All of which is simply to say that the present report does not pretend to be complete. It may serve, however, to call attention to the simplicity and convenience of a method which makes possible a more thorough investigation of the problem than has heretofore been possible and thereby stimulate further study.

The problem is of especial importance at the present time. In war medicine the use of opiates cannot always be as carefully safeguarded as in private practice, surgical care may, of necessity, be delayed and opiates in larger doses and over longer periods of time than usual have to be administered. This increases the danger of drug addiction. Merely from the standpoint of conserving present stocks of opiates, the question of their potentiation is important.

SUMMARY

A convenient and reasonably accurate method for assaying the analgesic potency of drugs is described and data indicating its reliability are presented. This method was applied to the determination of the analgesic properties of certain drugs and drug combinations, with the following results;

1. Little analgesia is conferred by the ordinary analgesics, (aminopyrine, aspirin, etc.) either alone or in combination, unless very large doses are given. Of these aminopyrine was most effective.

2. Little analgesia is conferred by barbiturates, either alone or in combination with the above group.

3. A considerable degree of potentiation of the analgesic properties of morphine and codeine is obtained by using aminopyrine, cyclopal (a barbiturate) either alone or, better, together, in combination with the opiate. This potentiation is greater when the agents are given intraperitoneally than orally. Magnesium sulfate with cyclopal is also effective

4. It is quite possible that even better results might be achieved with other combinations or dosages.

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TOXICITY OF ACETOIN

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An increase in the rate of disappearance of alcohol from the blood following injection of insulin and glucose has been repeatedly reported (1–15). Goldfarb, Bowman and Parker (16) have found that in men there is marked recovery from deep intoxication following this injection. The attendant decrease in concentration of alcohol which they report is so great that considerable increase in metabolism would result if the alcohol were oxidized. They recorded no evidence of such heat liberation. More recently, Westerfeld, Stotz and Berg (17) have found that in dogs which had received alcohol, the administration of pyruvate caused a marked increase in the rate of disappearance of alcohol from the blood. They interpret their results as indicating that acetaldehyde, produced by a primary oxidation of alcohol, may be condensed with pyruvate to form acetoin. Such a reaction would provide a relationship between the metabolism of alcohol and of carbohydrate. The condensation of acetaldehyde and pyruvate utilizes no oxygen and, if it occurred following the injection of insulin and glucose, would afford an explanation for the reduction in the concentration of alcohol without a large production of heat. There remains, however, the question of the physiological effects of the acetoin which would be formed. Alcohol would produce 1.9 times its weight of acetoin. Greenberg (18) has shown that acetoin, when injected intravenously, does not disappear from the blood more rapidly than alcohol.

To explain the clinical improvement observed by Goldfarb, Bowman and Parker (16), on the basis of the formation of acetoin, would necessitate that any intoxicating action of acetoin should not be an addition to that of the alcohol. Except for the observation of Neuberg and Gottschalk (19) that injection of 1 or 2 cc. of acetoin caused no ill effects in a rabbit, no study has been made of the physiological action of acetoin.

In a previous study (18) on the disappearance of acetoin from the blood, it was found that the injection of 2 grams of acetoin per kilo into a dog was followed by greater intoxication than injection of a similar amount of alcohol. This feature was further investigated, as reported here, using rats and following exactly the procedure described by Haggard, Greenberg and Rakieten (20) for ethyl alcohol. The acetoin was diluted to 30% with water and very small amounts were injected intraperitoneally at frequent intervals to assure complete absorption and at the same time to permit distribution to keep pace with absorption. In one series of 20 rats, the injections were continued until the animals were intoxicated just to the point of loss of the "righting reflex"; heart blood was then drawn and the concentration of acetoin determined. With an equal number of rats the same procedures were carried out with ethyl alcohol instead of acetoin. The

concentrations obtained are given in the accompanying figure. The values for acetoin ranged from 227 to 251 mgm. % with an average of 235. The corresponding figures for alcohol were 288 to 312 mgm. % with an average of 300. Acetoin, in producing the symptoms of intoxication, is about one and a quarter times as active as alcohol. The intoxication from acetoin resembles in every respect that from alcohol.

In another series of 20 rats, the injection of acetoin was continued until respiratory failure had occurred; blood was then drawn from the jugular vein (20) and the concentration of acetoin determined. With an equal number of rats

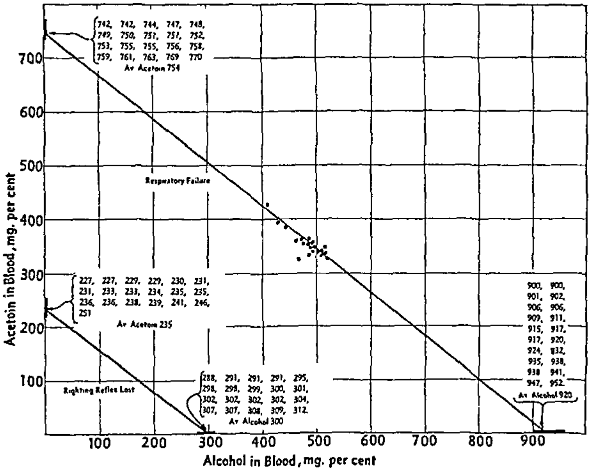

FIG. 1. CONCENTRATIONS OF ACETOIN AND ALCOHOL IN THE BLOOD AT LOSS OF RIGHTING REFLEX AND AT RESPIRATORY FAILURE

the same procedure was carried out with alcohol. The concentrations obtained are given in the accompanying figure. Those for acetoin ranged from 742 to 770 mgm. % with an average of 754. The corresponding figures for alcohol were 900 to 952 with an average of 920. The findings for alcohol are in agreement with those obtained by Haggard, Greenberg and Rakieten (20). Acetoin, in producing respiratory failure, is about one and a quarter times as active as alcohol. This ratio agrees with that found for the loss of the righting reflex.

In a final experiment, both alcohol and acetoin were given to 20 rats until respiratory failure occurred; the concentrations of both substances were then

determined in the blood. The alcohol was given first by slow injection until a total of 5 grams per kg. had been administered; the injection of acetoin was then started and continued until respiratory failure. The analysis for alcohol was made by precipitating the blood protein with tungstic acid, distilling and determining the alcohol in the distillate by oxidation with dichromate after the method of Harger (21). Acetoin reacts quantitatively with dichromate (22) as does diacetyl formed by the oxidation of acetoin; it was therefore necessary to correct for the amount of this substance present. Ten cc. of 50% ferric chloride were

TABLE 1

Determination of alcohol and acetoin in blood

Solution containing: Acetoin	Solution containing: Alcohol	Acetoin found	Error	Alcohol found	Error
mgm. %	*mgm. %*	*mgm. %*	*mgm. %*	*mgm. %*	*mgm. %*
50	225	48.9	−1.1	222	−3
		49.5	−0.5	223	−2
		49.3	−0.7	226	+1
		50.2	+0.2	225	0
		48.8	−1.2	224	−1
		48.7	−1.3	223	−2
		49.3	−0.7	226	+1
		50.1	+0.1	225	0
		48.8	−1.2	223	−2
		50.1	+0.1	223	−2
Averages		49.4		224	
225	50	224.1	−0.9	49	−1
		223.5	−1.5	50	0
		224.8	−0.2	50	0
		223.6	−1.4	51	+1
		224.2	−0.8	49	−1
		223.7	−1.3	50	0
		225.6	+0.6	48	−2
		224.1	−0.9	49	−1
		223.9	−1.1	50	0
		224.5	−0.5	49	−1
Averages		224.2		49.5	

introduced into the distillation flask with the blood filtrate and the flask was immersed in a hot water bath for 30 minutes prior to distillation. The acetoin was thus converted into diacetyl (18). The distillate was divided into two portions; one was subjected to the usual dichromate oxidation and in the other the concentration of diacetyl was determined with the polarograph; the concentration found yielded that of the acetoin present in the blood (18). The amount of dichromate which could react with the amount of diacetyl present was subtracted from that used in the titration of the distillate containing both alcohol and diacetyl; the remainder gave the correct concentration of alcohol. Table 1

gives the results of 20 analyses by this procedure made on mixtures of known amounts of acetoin and alcohol.

The error in determining the alcohol ranged from +1 to −3 mgm. % in a solution containing 225 mgm. % and 50 mgm. % of acetoin; and +1 to −2 mgm. % in a solution containing 50 mgm. % of alcohol and 225 mgm. % of acetoin. In the first solution, the error in determining acetoin was +0.2 to −1.3 mgm. % and, in the second, +0.6 to −1.5 mgm. %.

As seen from figure 1 in which the concentrations of acetoin and alcohol found in the blood at respiratory failure are plotted against each other, the action of the two substances is directly additive. The facts, first, that acetoin is more intoxicating than alcohol, and second, that alcohol would, on oxidation to acetaldehyde and condensation with pyruvate, yield nearly twice its weight of acetoin, do not support the suggestion that the clinical improvement in intoxication following injection of insulin and glucose is due to the formation of acetoin.

CONCLUSIONS

1. Intoxication from acetoin resembles that from alcohol. Acetoin is approximately 25% more active in producing intoxication and respiratory failure than is alcohol.

2. The intoxicating and lethal action of alcohol and acetoin, when administered together, are additive in proportion to their respective activities.

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PHARMACOLOGICAL PROPERTIES OF SIMPLE COMPOUNDS OF HISTAMINE WITH AMINO ACIDS

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In previous reports we have studied the pharmacological properties of trypsin, as well as its histamine-liberating activity (1, 2, 3). Other proteolytic enzymes have been found to produce a similar liberation of histamine, as shown by the increase of capillary permeability of the rabbit skin (4). Among those proteolytic enzymes papain, ficin and chymotrypsin were found to produce a blue spot around the injected area of the rabbit skin when trypan blue is injected into the vein. Notwithstanding that, chymotrypsin (5) displays little or no pharmacological activity when tried *in vitro* upon the isolated guinea pig gut or *in vivo* upon the arterial blood pressure of the cat. This fact might be understood as indicative of a slow liberation of histamine by chymotrypsin, which stands in contrast to the sudden and conspicuous liberation of histamine by trypsin. Although we did not make an exact quantitative comparison between the pharmacological effects of trypsin and chymotrypsin, the differences are co conspicuous that they could not be explained on a basis of differences in proteolytic activity of both enzymes upon a protein substrate like casein.

The linkages of histamine with the cell constituents are of unknown nature, but the liberation of histamine by proteolytic enzymes might suggest a peptide linkage between the amine and the amino acid chains of proteins inside the cells. Furthermore, the difference of activity between trypsin and chymotrypsin might well suggest a certain specificity in this linkage. To attack the problem, we have synthesized several simple compounds of histamine with amino acids (6), in which histamine is bound to the carboxyl groups of the amino acid residues.

The contact with several proteolytic enzymes (beef spleen and swine kidney cathepsin, and papain) resulted in a definite splitting of the compound as shown by the estimation of free carboxyl groups (6).

The following report shows that histamine is present in such compounds, in an inactive form, as it may be normally in living tissues. Acid hydrolysis liberates this bound histamine and produces free histamine, active upon smooth muscles and the circulatory apparatus of the cat.

Material and methods. We have studied the following compounds: I) acetyldehydrophenylalanyl histamine, II) acetyl *d, l* phenylalanyl histamine, III) benzoyl *l* tyrosyl histamine, IV) carbobenzoxy *l* tyrosyl histamine and V) carbobenzoxy *l* leucyl histamine. Although the synthesis of these compounds will be described in detail in a later publication (6), we wish to present here an outline of the methods used.

Acetyldehydrophenylalanyl histamine. One equivalent of histamine dihydrochloride (1.84 gram) dissolved in 10 cc. water was treated successively with 20 cc. of normal NaOH, 10 cc. of acetone and one equivalent of the azolactone of acetylamino cinnamic acid. The mixture was shaken in a machine for 2 hours, and then dried *in vacuo*. The substance

crystallized easily from hot water. The hydrochloride was prepared by adding an equivalent of HCl; it crystallized well from hot ethyl alcohol.

Acetyl d, l phenylalanyl histamine. The preceding compound, in solution in methyl alcohol, was hydrogenated, with palladium black as a catalyser, in the presence of a small amount of acetic acid The solvent and the acetic acid were removed under reduced pressure, in the usual way.

Benzoyl l tyrosyl histamine. One equivalent of benzoyl *l* tyrosine hydrazide (0, 6 gram) was dissolved in 10 cc. water, 1.5 cc. concentrated HCl and 1 cc glacial acetic acid. The solution was then cooled in an ice-salt mixture and 0, 18 gram of sodium nitrite in 4 cc. of water was added in small portions. The resulting azide was extracted with ethyl acetate and washed with water, dilute $KHCO_3$ solution, and water, and made to react with a solution of two equivalents of histamine (0, 44 gram) free base in ethyl acetate. The next day the ethyl acetate was removed by evaporation *in vacuo* and the whole precipitate was taken up in boiling water On cooling a precipitate was formed and purified by successive recrystallizations.

Carbobenzoxy l tyrosyl histamine. The starting material was carbobenzoxy *l* tyrosine hydrazide (0, 64 gram) and the conditions and quantities of the reacting mixtures were the same as indicated for the preparation of benzoyl *l* tyrosyl histamine.

Carbobenzoxy l leucyl histamine. Carbobenzoxy *l* leucine hydrazide (0.84 gram) was transformed into the corresponding azide and made to react with 2 equivalents of histamine free base (0 66 gram) in ethyl acetate. The crystallization followed from hot water, in the same way as for the other two similar compounds.

The methods described above are probably sufficiently general to allow the synthesis of other compounds containing histamine bound to amino acids. The five compounds synthesized constituted, however, a number well suited for our purpose. Their physical and chemical properties are summarized in table 1.

The compounds were dissolved in saline, after the addition of an equivalent amount of hydrochloric acid. For the experiments on the guinea pig gut, the solution was accurately neutralized and made up to the pH of Tyrode solution; thymol blue was the indicator. Acid hydrolysis was performed by treating 10 mgm. of the compound with 5 cc. of concentrated hydrochloric acid in an appropriate amount of water. The mixture was then boiled for one hour, the acid evaporated *in vacuo* by three successive additions of ethyl alcohol. The crystalline residue was taken up in distilled water and carefully neutralized with normal NaOH.

The assay on the isolated guinea pig gut was performed in the usual way, in a Dale's apparatus. The guinea pig gut was carefully washed out through the lumen, with warm Tyrode solution and an approximately 1 inch fragment suspended in Tyrode, without the addition of atropine.

RESULTS. *Effects on the blood pressure of the cat.* Fig. 1 shows one experiment made with carbobenzoxy *l* leucyl histamine, injected intravenously in a cat. At 10, histamine (1 γ of the base) was injected; at 11, carbobenzoxy *l* leucyl histamine (2 mgm.) before hydrolysis and at 12, the same compound (2 mgm.) after hydrolysis, were injected. We can see that the effect of 2 mgm. of carbobenzoxy *l* leucyl histamine was less pronounced than the effect produced by 1 γ of histamine. After hydrolysis, however, the injection of a corresponding amount produces a profound shock, considerably more severe than the one produced by 0 2 mgm. of histamine base.

Fig. 2 shows the effect of 1 γ of histamine (*1*), 2 mgm. of carbobenzoxy *l* leucyl histamine (in *2*), 2 mgm. of carbobenzoxy *l* tyrosyl histamine (in *3*) and 2 mgm. of benzoyl *l* tyrosyl histamine (in *4*). As we can see the effect produced by all

TABLE 1

COMPOUND	FORMULA	MOLECULAR WEIGHT	MELTING POINT	SOLUBILITY IN WATER
I. Acetyldehydrophenylalanyl histamine	$C_6H_5CH{=}C\cdot CO{-}NHCH_2CH_2C{=}CH$ $NH\cdot COCH_3$ $\quad$ N $\quad$ NH CH	334.8	134–137°	Base less soluble. Hydrochloride very hygroscopic
II. Acetyl *d, l* phenylalanyl histamine	$C_6H_5CH_2CH\cdot CO{-}NHCH_2CH_2C{=}CH$ $NH\cdot COCH_3$ $\quad$ N $\quad$ NH CH	336.8	95–100°	Almost deliquescent as hydrochloride
III. Benzoyl *l* tyrosyl histamine	$HOC_6H_4CH_2CH\cdot CO{-}NHCH_2CH_2C{=}CH$ $NH\cdot COC_6H_5$ $\quad$ N $\quad$ NH CH	378.4	140–146°	Very soluble as hydrochloride
IV. Carbobenzoxy *l* tyrosyl histamine	$HOC_6H_4CH_2CH\cdot CO{-}NHCH_2CH_2C{=}CH$ $NH\cdot COOCH_2C_6H_5$ $\quad$ N $\quad$ NH CH	408.4	147	Almost insoluble as base, very soluble as hydrochloride
V. Carbobenzoxy *l* leucel histamine	$(CH_3)_2{>}CHCH_2CH\cdot CO{-}NHCH_2CH_2C{=}CH$ $NH\cdot COOCH_2C_6H_5$ $\quad$ N $\quad$ NH CH	358.4	113–117°	Fairly soluble as base

the three substances before acid hydrolysis is very slight, and might be attributed to traces of histamine still present as an impurity.

Fig. 3 shows the effect of benzoyl *l* tyrosyl histamine, before (at *1*) and after (at *2*) acid hydrolysis. In both cases an amount equivalent to 2 mgm. of the compound was injected. In 3, histamine (50 γ of the base) was injected.

Fig. 4 shows a similar experiment performed with acetyldehydrophenylalanyl

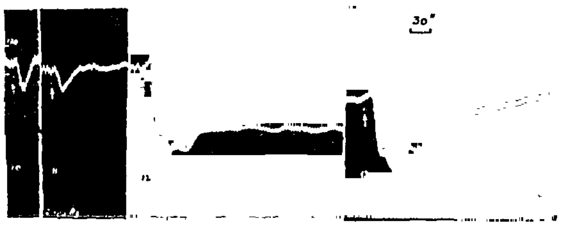

FIG. 1. BLOOD PRESSURE RECORD IN THE CAT
Experiments with carbobenzoxy *l* leucyl histamine. Explanation in the text

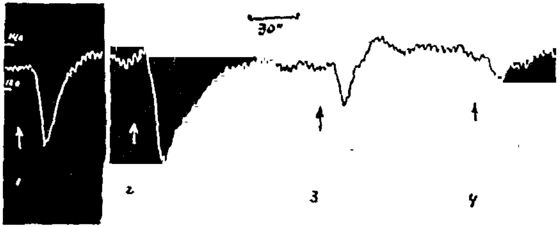

FIG. 2. CAROTID BLOOD PRESSURE IN THE CAT
(*1*) $l\gamma$ of histamine, (*2*), (*3*) and (*4*) respectively 2 mgm. of carbobenzoxy *l* leucyl histamine, of carbobenzoxy *l* tyrosyl histamine and of benzoyl *l* tyrosyl histamine.

FIG 3. CAROTID BLOOD PRESSURE IN THE CAT
Experiments with benzoyl *l* tyrosyl histamine. Explanation in the text

histamine. In (*1*), 2 4 mgm. of the compound before hydrolysis and in (*2*) an amount corresponding to 0.5 mgm. of the compound after hydrolysis were injected. In (*3*), 0.2 mgm. of histamine base was injected.

Estimation of the histamine liberated by acid hydrolysis. Fig. 5 shows an estimation of the histamine content of the hydrolysate of acetyldehydrophenylalanyl histamine, by assaying on the blood pressure of the cat. In *5*, *6* and *7* respectively 1 γ of histamine base, 0.6 cc. of a solution containing 5 γ per cc. of the acetyldehydrophenylalanyl histamine after hydrolysis and 0.8 γ of histamine were injected. The calculated amount of free histamine should be 1.6 γ

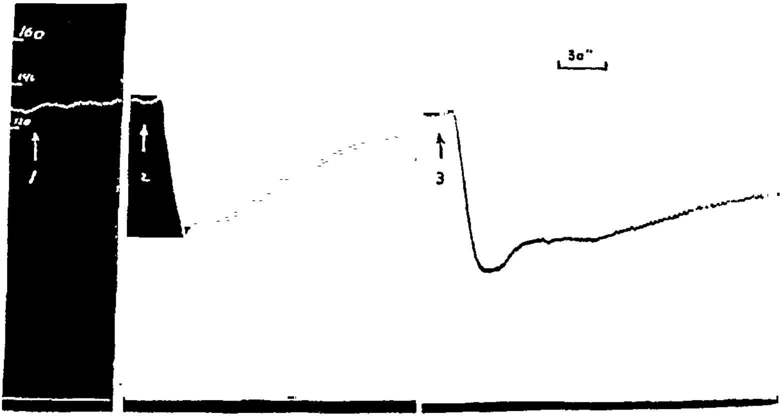

FIG. 4. CAROTID BLOOD PRESSURE IN THE CAT
Experiments with acetyldehydrophenylalanyl histamine. Explanation in the text

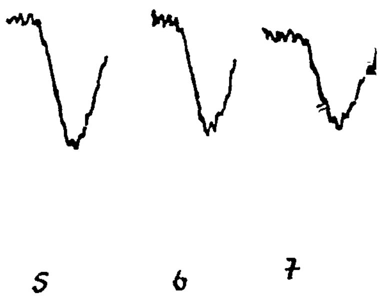

FIG. 5. ASSAY ON THE BLOOD PRESSURE OF THE CAT
Estimation of histamine liberated after hydrolysis of acetyldehydrophenylalanyl histamine

of histamine per cc., while the value found by the biological assay equals approximately to 1.4 γ of histamine base per cc. The values are so close that one might conclude that almost all the histamine previously bound to the amino acid residue is set free after acid hydrolysis.

Experiments on the guinea pig gut. The results obtained on the guinea pig gut by testing the above compounds, before and after hydrolysis, quite agree

with the experiments made on the blood pressure of the cat. At *22* in figure 6 acetyldehydrophenylalanyl histamine (0.4 mgm.) before hydrolysis, was tested and did not produce any response. At *25* there was added to the bath a corresponding amount of the compound after acid hydrolysis, and a conspicuous and lasting response of the muscle was produced. At *20*, *21*, *23*, *24*, *26*, *27* and *28*, the same amount of histamine was added (0.04 γ). We can also see that after the response to the hydrolysate (at *25*), the same amount of histamine elicited a smaller response, showing that the muscle was made partially refractory to this small amount of histamine.

Experiments on the human skin. Results perfectly parallel to those obtained previously, were obtained by testing on the flare produced by the histamine liberated after acid hydrolysis. Before hydrolysis, the compounds usually pro-

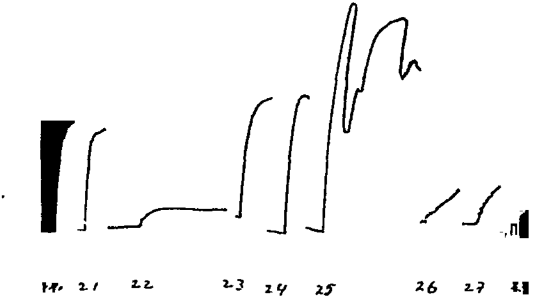

FIG. 6. EXPERIMENTS WITH THE GUINEA PIG GUT
Effect of acetyldehydrophenylalanyl-histamine, before (*22*) and after (*25*) acid hydrolysis

duced only a very slight response in appropriate concentrations; after hydrolysis, however, the same concentration produced conspicuous responses.

In vivo experiments on the guinea pig. The compounds before hydrolysis were almost harmless for the guinea pig. We injected intravenously as much as 20 mgm. of acetyldehydrophenylalanyl histamine without producing any symptom. The injection of 1 to 2 mgm. of the hydrolysate, however, killed the guinea pigs with the typical symptoms of histamine poisoning.

DISCUSSION. The nature of the binding of histamine with the cell constituents is still unknown but one might assume that histamine is present in the cells, forming peptide bonds with the amino acid chains which constitute the protein part of the cells. The fact that histamine, when entering in chemical combination with amino acid residues, loses its pharmacological properties, is very interesting in view of the fact that histamine is present in the cells in an inactive form. The above compounds, might represent a *model* of the so-called bound

histamine, which is changed into free histamine under the action of irritants, proteolytic enzymes, animal poisons, etc.

It is, however, worthy of note that these compounds do not reproduce all the features of bound histamine. For instance, the simple interaction of protein precipitants, as trichloracetic acid, is not enough to set free histamine bound to these amino acid residues. The linkage of histamine in the studied compounds is not as loose as the linkage of histamine with the cell proteins, but one has to consider that we have only *models*, and by no means the true connections existing between histamine and cell proteins. The loose histamine linkage in living cells might depend, for instance, on the length of the amino acid chain connected to histamine. The chances are that a long polypeptide chain, with histamine anchored to one of the ends, is more likely to behave toward heating and protein precipitants as living proteins do. Henceforth, the chemical problem would consist in an attempt to increase the number of amino acids in the peptide chain connected to histamine.

Recently Feldberg (7) assumed that the release of histamine by antigen in anaphylactic shock might be due to an activation of cellular proteinases, by exclusion of the anti-proteinases supposedly present in all living cells. It is interesting to note that the liberation of the histamine previously bound to the amino acid residues in the above compounds was performed by rupture of a peptide linkage as would happen in the living cell by activation of proteolytic enzymes. Consequently the study of simple models, like those presented in this paper, might throw light on the problem of histamine liberation in the living cell. The reverse process, the capture of histamine by a free carboxyl group in the protein molecule, might constitute a general procedure followed by the tissues to detoxify histamine. Although the existence of a histaminolytic enzyme—histaminase—was shown by Best and McHenry (8), there is a general agreement that this substance plays only a partial rôle in the detoxification of histamine when the latter is injected intravenously or released from the tissues. The simple capture of histamine, as shown in the experiments of Ahlmark, Kornerup and Tarras-Wahlberg (9) might also play a subsidiary rôle.

Thus, the reversibility of the histamine connections with the cell proteins might explain not only the discharge of histamine following activation of the proteolytic enzymes, but also the capture of histamine when the poison is present in excess in the intercellular fluids.

CONCLUSIONS

The methods of synthesis of a number of compounds of histamine and amino acids—acetyldehydrophenylalanyl histamine, acetyl *d*,*l* phenylalanyl histamine, benzoyl *l* tyrosyl histamine, carbobenzoxy *l* tyrosyl histamine and carbobenzoxy *l* leucyl histamine—are briefly described, as well as their chemical and physical properties. Their outstanding characteristic resides in the fact that histamine is bound to the amino acid residues, forming peptide bonds with the carboxyl groups.

A pharmacological study of those simple compounds and their hydrolysates,

upon the blood pressure of the cat, the guinea pig gut, the human skin and the intact guinea pig, is described. All the 5 compounds display very slight pharmacological activity or none at all, while the corresponding hydrolysates show the characteristic pharmacological effects of free histamine.

A general discussion of the problem of the liberation of histamine from, as well as of its capture by living structures is presented. The 5 studied compounds are suggested as *chemical models* for bound histamine.

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SULFAPYRIDINE BACTERIOSTASIS OF LACTOBACILLUS ARABINOSUS AND ITS COUNTERACTION[1]

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West (1) found that sulfapyridine inhibited the usual curative effect of nicotinic acid on dogs rendered deficient on a blacktongue diet and that the feeding of fresh liver together with the sulfapyridine gave normal weight responses. He concluded that sulfapyridine might inhibit the action of nicotinic acid but not that of preformed coenzymes. The experimental observations of West have been confirmed by Schaefer, McKibbin and Elvehjem (2) who used a more highly purified basal ration. In addition to comparing the effectiveness of nicotinic acid and fresh liver they also fed equivalent amounts of dried liver and nicotinamide and found them to be inactive. In order to extend these studies, we decided to use the organism *Lactobacillus arabinosus 17-5* because nicotinic acid is a distinct growth factor for this organism and because we had had considerable experience with the organism in the microbiological determination of nicotinic acid.

A number of bacterial studies on the relationship between sulfonamides and nicotinic acid metabolism have been reported. West and Coburn (3) found that sulfapyridine prevented the growth promoting effect of nicotinic acid on *Staphylococcus aureus* in a nicotinic acid-deficient medium. The addition of coenzymes gave excellent growth. Straus, Dingle and Finland (4) were unable to confirm these results, but Spink, Vivino and Mickelson (5) have recently reported that the counteracting effects of cozymase can be demonstrated if experimental conditions are properly controlled. Dorfman (6) has noted that sulfapyridine inhibits the increased respiration of dysentery bacilli caused by nicotinamide, while McIllwain (7) has shown that sulfonic acid derivatives of nicotinic acid added to cultures of *Staphylococcus aureus* in a synthetic medium inhibit the growth promoted by nicotinic acid, nicotinamide and cozymase.

In our investigations, we first studied the bacteriostatic effect of sulfapyridine and its counteraction by *p*-amino benzoic acid. In an attempt to correlate the effects of sulfapyridine on blacktongue dogs with its effect on the organism, some of the same fresh and dried livers used by Schaefer *et al.* in the dog studies were tested in our bacterial studies for their counteracting potency. Fractions prepared from liver extract were also tested. With the hope of learning something about the mechanism by which sulfapyridine interferes with nicotinamide-containing coenzyme systems, the counter-acting effects of various levels of

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nicotinic acid, nicotinamide-ribose nucleoside, nicotinamide and cozymase were studied.

EXPERIMENTAL. *Medium used for culture of Lactobacillus arabinosus 17-5.*

Acid hydrolyzed casein	0.50%
Tryptophan	0.01%
Cystine	0.01%
Glucose	1.00%
Sodium acetate	0.60%
Adenine	1.0 mg. %
Guanine	1.0 mg. %
Uracil	1.0 mg. %
Thiamine	0.1 p.p.m.
Pantothenic acid	0.1 p.p.m.
Pyridoxine	0.1 p.p.m.
Riboflavin	0.2 p.p.m.
Biotin	0.4 parts per billion
K_2HPO_4	500 p.p.m.
KH_2PO_4	500 p.p.m.
$MgSO_4 \cdot 7H_2O$	200 p.p.m.
NaCl	10 p.p.m.
$FeSO_4 \cdot 7H_2O$	10 p.p.m.
$MnSO_4 \cdot 4H_2O$	10 p.p.m.

General procedure. The general procedure employed was much the same as that outlined by Snell and Wright (8) for the microbiological determination of nicotinic acid. The medium was made up in double strength, 5 cc. were pipetted into each of a number of 6″ by ⅝″ bacteriological test tubes and the desired levels of sulfapyridine, *p*-amino benzoic acid and nicotinic acid were added in water solution. Unless otherwise specified, the other supplements were added after the medium had been sterilized. Distilled H_2O was then added to each tube in such quantity that after the final supplements had been added the total volume of liquid in each tube was 10 cc. The tubes were plugged with cotton and autoclaved for 20 minutes at 15 lbs. pressure. After cooling the tubes at 37°C., the additional supplements were added and the tubes were inoculated with one drop of a 24 hour culture of *Lactobacillus arabinosus 17-5* grown in 10 cc. of the basal medium to which 1γ of nicotinic acid has been added. The tubes were then incubated at 37°C. and the lactic acid produced in 48 hours was titrated with 0.1N NaOH. Supplementary visual observations of turbidity were made at 24 and 48 hours.

Sterilization of supplements. The sterilization of supplements was carried out by passing solutions (or suspensions of homogenized material, in the case of liver) through a Seitz filter. These preparations were added to the tubes by sterile pipettes. To prevent the marked changes in pH which might occur in the filtration procedure (9) the solutions were kept slightly acid with phosphate buffer. As will be shown later, there was no indication of any loss of activity in the solutions on passage through the filter. To check on the sterility of the solutions, uninoculated control tubes were run in all cases.

Effect of sulfapyridine. Preliminary experiments showed that with the medium described above, plus nicotinic acid, a concentration of 1γ of sulfapyridine per cc. of medium was sufficient for bacteriostasis. However, a slight amount of growth always took place before bacteriostasis occurred, a phenomenon which is known to be characteristic of the sulfonamides (10). In the absence of sulfapyridine, the addition of 0.1γ of nicotinic acid per cc. of medium allowed the production of 9 to 10 cc. of 0.1N acid per tube. One γ of sulfapyridine per cc. of medium added with the nicotinic acid allowed the production of only 1 cc. of 0.1N acid.

Counteracting effect of p-amino benzoic acid. The bacteriostatic effect of 1γ of sulfapyridine per cc. was completely overcome by 0 001γ of p-amino benzoic acid per ml. of medium. When the level of sulfapyridine was raised to 10γ or 100γ per cc. of medium, the amount of p-amino benzoic acid necessary for counteraction was correspondingly increased so that the ratio remained constant. The molar ratio of p-amino benzoic acid to sulfapyridine was roughly 1/500. The existence of a constant ratio between the p-amino benzoic acid and sulfonamides has been observed by a large number of workers using a variety of organisms (11, 12, 13).

Counteraction of sulfapyridine by fresh and dried livers. It was hoped that the difference in potency between fresh and dried liver in the counteraction of sulfapyridine in blacktongue dogs could also be demonstrated in the case of the organism, so that a correlation of results might be possible. However, such was not the case. Although in preliminary studies in which turbidity observations were used several samples of fresh liver appeared to be more active than equivalent amounts of dried liver, more careful studies, in which both turbidity observations and measurement of acid production were employed, showed that there was no measurable difference between the two. One γ of sulfapyridine was *counteracted by 50γ of dried liver or an equivalent amount (150γ) of fresh liver.*

To determine whether or not activity was lost in filtration through the Seitz filter, samples were also homogenized in sterile H_2O and added to the sterilized medium directly. Turbidity was observed up to the time that there was visible growth in the control tubes. This technique, though somewhat unsatisfactory, would have demonstrated any marked differences in potency between the filtered and unfiltered solutions. No differences could be detected.

The fresh livers showed the same potency whether they were added to the medium immediately after removal from the animal or stored for varying lengths of time at temperatures varying from 0° to 37°C. Rat, calf, and hog livers were tested. Some of the livers were the same ones used in the dog studies by Schaefer *et al.* The heat-labile material in fresh liver which is active in the case of the dog might also be active in the case of the organism, but due to differences in metabolism its effect on the organism might be masked by other counteracting substances.

Fractionation of the active material in liver extract. A number of fractions of liver extract which Black, Overman, Elvehjem and Link (14) were using in rat studies involving sulfaguanidine were available to us and were tested for their counteracting potency. The relative potencies are summarized in table 1. The results on treatment with acid, acid ether extraction and calcium precipitation are at variance with what one might expect from the known properties of p-amino benzoic acid, if that were the factor accounting for most of the activity in liver extract. It is interesting to note that Black *et al.* found the preparations to have the same relative potencies in counteracting the effect of sulfaguanidine on rats that we found in our bacterial studies with sulfapyridine.

Further studies were made with solubilized liver extract and grass juice. These materials and crystalline p-amino benzoic acid were autoclaved at 15 pounds pressure for 1.5 hours with 1 N HCl. No activity was lost in the case of p-amino benzoic acid but 50% of the activity in the solubilized liver extract and 80% of the activity in the grass juice was destroyed. Since the properties demonstrated in Table I are similar to those reported for folic acid preparations, concentrates of folic acid from solubilized liver extract and grass juice were tested. The concentrates were prepared according to the method of Hutchings, Bohonos and Peterson (15). The procedure was carried through the second elution from superfiltrol. Both preparations demonstrated from 20–30% of the activity of the starting material. Autoclaving with 1 N HCl for 1 5 hours at 15 pounds destroyed 60–70% of the activity of the concentrate from solubilized liver extract but no destruction could be measured in the concentrate from grass juice In the case of the latter, there may be a concentration of "bound" p-amino benzoic acid which is liberated by acid treatment and thus conceals the concentration of the acid labile factor. At any rate, there is no doubt of the presence of acid labile counteracting material in the original liver extract or grass juice.

The acid-ether insoluble fraction we have encountered in liver extract differs from a similar fraction found in enzymatically digested casein by MacLeod (16) in that our fraction is quite stable to treatment with concentrated alkali. Loomis, Hubbard and Neter (17) reported an active acid-ether insoluble fraction in yeast but did not study its stability to acid and alkali.

TABLE 1

Effectiveness of fractions of liver extract in counteracting bacteriostatic effect of sulfapyridine

	0.5 γ nicotinic acid and 1.0 γ sulfapyridine added per cc. of medium amounts of original liver extract represented by added supplements			
	0.025 *mgm./cc.*	0.05 *mgm./cc.*	0.10 *mgm./cc*	0.50 *mgm./cc.*
Liver extract	++	+++	+++	+++
Neutral autoclaved liver extract	++	+++	+++	+++
Acid autoclaved liver extract	−	−	+	++
Alkaline autoclaved liver extract	+	++	+++	+++
Norite filtrate of liver extract	−	−	−	±
Norite eluate of liver extract	+	++	+++	+++
Calcium precipitate of liver extract	+	++	+++	+++
Calcium filtrate of liver extract	−	−	−	±
Acid ether extract of liver extract	−	−	−	+
Acid ether residue of liver extract	+	++	+++	+++
Dialysate of liver extract	++	+++	+++	+++
Butanol extract of liver	−	−	−	±

−, no growth; ±, very slight growth; +, fair growth; ++, good growth; +++, excellent growth.

TABLE 2

Potency of various compounds in counteracting sulfapyridine bacteriostasis of lactobacillus arabinosus

Supplement	0.5 γ nicotinic acid and 2.0 γ sulfapyrdine added per cc. of medium amount of supplement added per cc. of medium								
	0.001 γ	0.1 γ	0.5 γ	1 γ	5 γ	10 γ	25 γ	100 γ	400 γ
Nicotinic acid	−	−	−	−	−	−	±	++	+++
Nicotinamide	−	−	+	++	++	±	−	−	−
Coenzyme I	−	−	−	+	++	++	++		
Nicotinamide-ribose nucleoside	−	−	−	+	++	++	++		
P-amino benzoic acid	++	+++	+++	+++	+++	+++	+++	+++	+++

−, no growth; ±, slight growth; +, fair growth; ++, good growth; +++, excellent growth.

Results. *Counteraction of sulfapyridine by nicotinic acid and nicotinamide compounds.* Results of the studies involving nicotinic acid and nicotinamide compounds are given in table 2.

In the absence of sulfapyridine, 0.05 γ of nicotinic acid per cc. of medium gave

excellent growth. In the presence of 2 γ of sulfapyridine per cc. levels of nicotinic acid from 0.001 to 10 γ per cc. had no effect. When the nicotinic acid concentration was raised to 100 γ per cc. the bacteriostasis was completely counteracted. It is possible that the effects of these high levels were due to contamination with *p*-amino benzoic acid. No toxic effects were observed when the nicotinic acid concentration was increased to 1 mgm. per cc. In all the other studies nicotinic acid was added at a level of 0.5 γ per cc. to provide an excess of this growth factor.

Nicotinamide was active at much lower levels than was the free acid, but it never completely counteracted the sulfapyridine. With 0.5 γ of nicotinamide per cc. there was some growth, and 1 to 5 γ per cc. provided fairly good growth. However, when amounts above 10 γ per tube were added bacteriostasis again occurred. This lack of counteraction by higher levels of nicotinamide may be due to a competition of nicotinamide with some other essential metabolite for a specific enzyme surface, in the same manner that sulfonamides presumably compete with *p*-amino benzoic acid (11). Mann and Quastel (18) have shown that high levels of nicotinamide completely inhibit the enzyme, in fresh brain tissue, responsible for the breakdown of cozymase. The inhibition produced by the high levels of nicotinamide could not be demonstrated in the absence of sulfapyridine, which indicates that sulfapyridine brings about an abnormal metabolism or in some other way sensitizes the organism to nicotinamide.

Studies with cozymase made it apparent that amounts in considerable excess of actual growth requirements were necessary to demonstrate counteracting effects. There may be several reasons for this. The cells may be only slightly permeable to the cozymase molecule or the cozymase and sulfapyridine may compete directly for the same enzyme surface. Their relative affinities would determine the amount of cozymase necessary for counteraction. Thus it is possible that sulfapyridine does not interfere with the synthesis of cozymase but that the normal amount synthesized is not great enough to compete successfully with sulfapyridine. At any rate, the addition of 1 γ of cozymase per cc. allowed fairly good growth, though the counteraction was never complete. Nicotinamide and cozymase showed about the same order of activity and the requirement for counteraction was more than ten times the growth requirement in the absence of the drug.

The possibility remained that the nicotinamide-ribose nucleoside might enter the cells more rapidly than cozymase and be converted to the coenzyme by the cells. Schlenk (19) reported the preparation of the nucleoside from cozymase by treatment with an almond phosphatase. Some of the enzyme was kindly supplied by Dr. Schlenk and several samples of cozymase were treated with the enzyme. In each case, the potency of the preparation was several times greater than that of the same amount of enzyme and cozymase added directly to the tubes. Later experiments with the nucleoside itself indicated that it has approximately the same order of activity as nicotinamide or cozymase. Hence the increased potency of the enzyme-treated cozymase must have been due to some other products or combination of products.

A preparation of the nucleoside itself was obtained from Dr. Schlenk. He found this preparation to be 60% pure by spectrophotometric analysis. This percentage of nucleoside accounted for all of the nicotinic acid activity as measured by the microbiological method. The amounts of nucleoside given in table 2 are expressed as micrograms of nucleoside itself, assuming the preparation to be 60% pure. Results obtained with the nucleoside were similar to those obtained with cozymase. Levels above 1 γ per cc. exerted a counteracting effect, but the counteraction was never complete.

As indicated before, *p*-amino benzoic acid was able to counteract sulfapyridine completely. In order to minimize the possibility of contamination of materials with this highly active compound, all the materials tested except the nucleoside were obtained from at least two different sources.

It was found throughout these studies that although the general effects of the various compounds were reproducible, they were not reproducible with the accuracy that may be obtained, for example, in the microbiological determination of nicotinic acid, in which case titrations are reproducible to 0.1 cc. Consequently general effects rather than specific titrations are presented in the tables. We believe that the variability encountered in our studies means that sulfapyridine exerts its bacteriostatic effects through a number of mechanisms and its effectiveness may be influenced by a large number of compounds. Therefore all the results reported were checked several times.

Action of sulfaguanidine and sulfasuxidine. Because of the recent use of sulfaguanidine and sulfasuxidine in animal studies we thought it was of interest to study their action on *Lactobacillus arabinosus.* Each of these drugs was found to be only about one-tenth as potent as sulfapyridine in producing bacteriostasis and both drugs were completely counteracted by *p*-amino benzoic acid even when the drugs were present at high levels. This is of interest because Welch (20) has reported that sulfasuxidine is not counteracted by *p*-amino benzoic acid in the rat. Several explanations might be proposed: (*a*) There might be accessory counteracting factors in our medium which are not present in the rat. (b) The effect of sulfasuxidine on different organisms may vary. (c) The drug may have effects on the rat which are distinct from the effect on intestinal bacteria, and these effects may not be counteracted by *p*-amino benzoic acid.

Discussion. The data presented give no direct evidence that sulfapyridine inhibits the synthesis of cozymase. Nicotinamide and cozymase are of about equal potency in counteracting sulfapyridine. Axelrod (unpublished data) was not able to demonstrate any effect of sulfapyridine on the *in vitro* synthesis of cozymase from nicotinic acid by red blood cells. It seems more likely that sulfapyridine inhibits the function of the coenzyme. Dorfman (21) has found that nicotinamide is able to reverse certain effects of sulfonamides which p-amino benzoic acid cannot. The possibility exists that the nicotinamide compounds might function *per se* in enzymatic processes. The relative potencies would depend on their affinities for the enzyme surface. It is also possible that sulfapyridine and cozymase are connected only indirectly, but there is no doubt that cozymase exerts a counteracting effect.

The inability of some workers to obtain sulfonamide counteraction with nicotinamide compounds may be due to the masking of sulfapyridine inhibition of nicotinamide-containing coenzyme systems by its effect on other systems in the case of the particular organism studied. The freeing of cozymase systems from inhibition might not be of great enough consequence to allow growth to take place. The necessity for proper levels of counteracting materials is shown in Table 2. Variation in media will lead to further difficulty in correlation of results obtained by different workers, since it is known that materials like methionine (22), peptones (15) and a specific sulfonamide inhibitor (23) can have a marked effect on sulfonamide studies.

Since adenine and guanine are present in our basal medium, it should be noted that these purines have been shown to affect sulfonamide action under certain conditions. Harris and Kohn (24), using *Escherichia coli*, found that guanine increases the methionine counteraction of sulfonamides, but in the absence of methionine it increases the activity of the drugs. Adenine increases the inhibition caused by sulfonamides both in the presence and absence of methionine. Martin and Fischer (25) have recently reported that adenine completely nullifies the sulfonamide protection against *Streptococcus hemolyticus* in the rat. *In vivo* studies furnish the organism with a very complex medium, so it is difficult to relate the two studies.

Since the exact mechanism of sulfapyridine's interference with bacterial growth is not clear, it is very difficult to theorize concerning its mode of action in the dog. Its effect might be exerted through the intestinal flora, in the intestinal mucosa, or in the tissues themselves. At any rate, the available evidence indicates that sulfapyridine does not function by preventing the formation of cozymase.

SUMMARY

1. Sulfapyridine prevents growth of *Lactobacillus arabinosus* and is completely counteracted by *p*-amino benzoic acid or high levels of nicotinic acid.

2. In contrast to results obtained in dog studies, fresh and dried liver are equally effective in counteracting sulfapyridine bacteriostasis.

3. A counteracting acid-labile factor (or factors) distinct from *p*-amino benzoic acid is present in liver extract and grass juice.

4. The non-*p*-amino benzoic acid fraction shows properties similar to those reported for folic acid preparations.

5. Nicotinamide compounds only partially counteract sulfapyridine and high levels of nicotinamide are inhibitory in the presence of the drug.

6. Sulfaguanidine and sulfasuxidine are less potent than sulfapyridine and are also completely counteracted by *p*-amino benzoic acid.

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A CONTRIBUTION TO THE PHARMACOLOGY OF PHENAZINE AND CERTAIN OF ITS DERIVATIVES[1]

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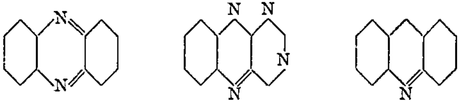
Owing to the occurrence of the phenazine nucleus in the bacterial pigment pyocyanin and in the pigment of *Chromobacterium Iodinum*, the pharmacology of phenazine is a matter of general interest. In addition, the structural relationship between phenazine and riboflavin and the existence of a reversible oxidation-reduction system "phenazine-dihydrophenazine" (1, 2) made a pharmacological study of this compound seem worth while. Besides, the recent shortage of quinine and the close structural relationship between phenazine and the acridine nucleus in Quinacrine (Atabrine) suggests the possibility of derivatives of phenazine possessing antimalarial activity.

Structural relations and physical properties. The following formulas show the relation between phenazine and other substances of marked pharmacologic



Phenazine — Cyclic Nucleus of Riboflavine — Cyclic Nucleus of Quinacrine

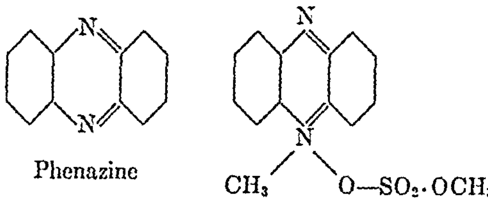
interest. Phenazine occurs in yellow crystals melting sharply at 171°C. Its solubility in water is of the order of magnitude of 1:3000. It is sparingly soluble in alcohol. It is refractory to reaction with many of the common laboratory reagents.

Toxicity studies. Intraperitoneal injections in rats of phenazine suspended in water containing tragacanth, in doses of 1 to 40 mgm. per 100 grams body weight, produced no grossly discernible toxic symptoms in 10 animals. Similar oral doses produced no toxic effects.

In a series of 40 rats phenazine was added to a basal diet of purina chow in concentrations of 0.1 to 1%. These animals were fed this diet over a period of 3 weeks. Many of the rats rejected the food containing phenazine, others ate liberally of it. The rats ingesting the food containing phenazine were sacrificed and complete histopathological studies were made on the important viscera. The lungs, liver and kidneys appeared normal. The mucous membranes of the alimentary tract did not appear irritated from the presence of phenazine in the food.

[1] The expense of the work was defrayed in part by a grant from Parke, Davis and Co., Detroit, Michigan

Convulsive action. The foregoing studies indicate that the insoluble parent compound, phenazine, is comparatively non-toxic to the rat, although upon prolonged feeding small percentages of phenazine in the diet caused the animals to reject their food. A simple derivative of phenazine, namely, *N*-methylphenazonium methyl sulfate was prepared for study. It is referred to generally as phenazine methosulfate. Its relationship to phenazine may be observed from the following formulas.



Phenazine

Phenazine Methosulfate

Intraperitoneal injections of phenazine methosulfate in rats elicited convulsive symptoms. Accordingly its antidotal value for typical barbiturate intoxication produced by fatal doses of pentobarbital sodium was investigated. The procedure as described previously (3) was employed. Phenazine methosulfate failed to serve as an antidote in 20 animals after LD-50 doses of the barbiturate in which picrotoxin was 100% effective. The minimal convulsive dose (20 animals) of phenazine methosulfate is 1.5 mgm. per 100 grams of rat. This dose was fatal to most animals. In 10 animals phenazine ethosulfate was observed to elicit convulsive seizures and to be equally as toxic as the metho compound. In mice, similar results were obtained with each compound. Pentobarbital or ether serves as an antidote for these convulsions. Glucose or calcium gluconate is without antidotal action. In 9 rabbits under paraldehyde anesthesia and arranged for measurements of their tidal respiratory volume, phenazine methosulfate failed to serve as an effective respiratory stimulant. An increased respiratory rate was observed in some animals. Large doses, however, produced convulsions and death without awakening the animal from the anesthesia. Pulmonary edema was observed in some instances. Rats frequently exhibit this effect after convulsive doses of phenazine methosulfate.

Blood pressure experiments. Dog. In doses of 5 mgm. intravenously, phenazine methosulfate produces an average rise in blood pressure of 40 mm. of mercury in 8 to 10 kg. dogs under pentobarbital or ether anesthesia. Tolerance was not readily acquired upon repeated doses. The minimal effective dose was 0.2 mgm. This pressor response is not abolished by atropine, and in the ergotaminized cat, exhibiting an epinephrine reversal, phenazine methosulfate produces a rise in blood pressure. That this effect is not the result of dimethyl sulfate itself, is shown by the fact that equimolar amounts of dimethyl sulfate do not influence the blood pressure of the dog, and samples of phenazine methosulfate possessed undiminished pressor activity after several recrystallizations. A typical pressor response in the dog under ether anesthesia is shown in figure 1.

Phenazine itself, in concentrations that could be obtained in hydroalcoholic solutions or inactive solvents suitable for injection purposes, failed to cause any blood pressure changes.

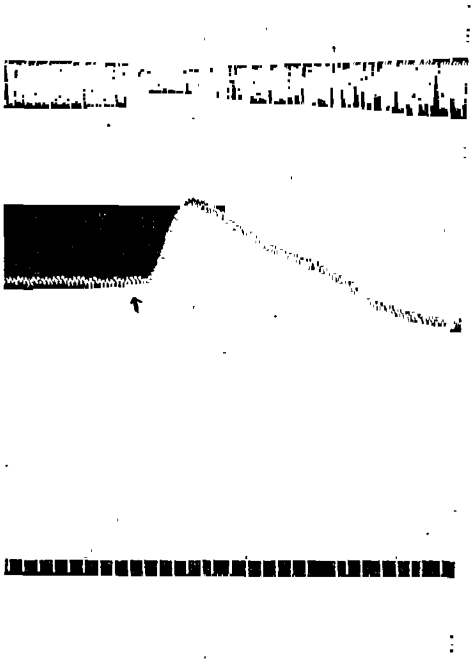


FIG. 1. THE EFFECT OF PHENAZINE METHOSULFATE ON THE BLOOD PRESSURE OF THE DOG (0 5 CC. 1 PER CENT SOLUTION I.V.)

Phenazine ethosulfate elicits a response similar to its homologue but to a lesser degree Massive doses of either compound produce death by cardiac arrest.

Dr. C. C. Pfeiffer of the laboratories of Parke Davis and Co. kindly tested phenazine methosulfate on the unanesthetized dog and observed the same type of pressor response as shown in fig. 1.

Analgesia—guinea pig. Through the courtesy of Dr. C. C. Pfeiffer experiments were conducted on guinea pigs using a modified Hardy-Wolff Apparatus to determine whether or not phenazine methosulfate produced analgesia. Two animals received 100 mgm./kg. and 2 others 200 mgm./kg. subcutaneously. In the first group one animal died of convulsions and both animals receiving the large dose died in this manner. There was no rise in pain threshold.

Influence on blood-sugar level. The striking character of the convulsive seizure produced by phenazine methosulfate and the pressor activity of the compound prompted a study of adrenergic effects. In normal fasting rabbits (10 animals) no significant changes in the blood-sugar level were observed after doses ranging from 50 mgm./kg. to the convulsive dose of 100 mgm./ kg. intraperitoneally. Blood samples taken by cardiac puncture from rats in convulsions produced by phenazine methosulfate were found to have a normal blood-sugar level.

Excretion. Rabbits and rats appear to either detoxify or excrete phenazine methosulfate and phenazine ethosulfate rapidly. Acutely convulsive doses are well tolerated when administered over long periods of time. The excreted urine

TABLE 1

Bactericidal activity of phenazine derivatives

	CONCENTRATION	*S. aureus*	*E. typhi*	
Phenazine	1:3000	+++	+++	
Metho-sulfate phenazine	1:100	+	+	Bactericidal
Etho-sulfate phenazine	1:100	+	+	Bacteriostatic
Methoxy phenazine	1:1000	+++	+++	
Neutral acriflavine	1:1000	+++		
Phenazine	Dry powder	+++		
Dihydro phenazine	Dry powder	+		Bacteriostatic

+++, no inhibition of growth; +, large zone of inhibition—35–40 mm.

of these animals contains red-colored compounds, presumably oxidized forms of phenazine methosulfate.

Bacteriological studies. Phenazine and certain of its derivatives were submitted to the standard F.D.A. test to evaluate bactericidal activity. *S. Aureus* and *E. typhi* were the organisms. The results are shown in table 1.

Antimalarial tests. Through the courtesy of Professor A. L. Tatum 2-methoxy-phenazine and 2-amino-phenazine were tested against avian malaria at the University of Wisconsin. In doses of 8–12 mgm. per day to canaries the compounds were ineffective.

SUMMARY

1. The experiments set forth in this communication give a first approximation of the pharmacology of phenazine and certain of its derivatives.

2. The phenazine nucleus appears to be quite inert as a pharmacologic agent, but its methosulfate exhibits a marked pressor response.

3. The soluble metho and ethosulfates of phenazine are mildly active against bacteria *in vitro*.

4. Preliminary antimalarial and analgesic tests on certain phenazine derivatives were negative.

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STUDIES ON VERATRUM ALKALOIDS

II. The Action of Veratridine and Cevine upon the Isolated Mammalian Heart[1]

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In the first publication of this series (1), concerning the action of veratrine hydrochloride upon the denervated heart-lung preparation of the dog, it was concluded that veratrine exerts an effect upon the isolated heart which in certain respects is similar to the action of the cardiac glycosides. Since the veratrine used for our previous studies was a mixture of several alkaloids, additional experiments were conducted to determine the activity of the pure alkaloids veratridine and cevine and of veratric acid.

The amorphous veratrum alkaloid now called veratridine was first isolated by Couerbe (2). Its chemical properties were later studied by Schmidt and Köppen (3) and particularly by Wright and Luff (4). Blount (5) established the correct empirical formula. According to Lissauer (6) the name veratridine was first used by Schmidt; it was, however, properly applied to the well-defined alkaloid for the first time by Blount. The base cevine was first isolated in amorphous form by Wright and Luff and was named by these authors. It was crystallized by Freund and Schwarz (7). According to Blount veratridine ($C_{36}H_{51}O_{11}N$) is the ester of cevine ($C_{27}H_{43}O_8N$) and veratric acid ($C_9H_{10}O_4$). The chemical structure of cevine is not fully established (8). Veratric acid is *3,4*-dimethoxy-benzoic acid.

Except in experiments on frogs and a few experiments on rabbits, for which Lissauer used the amorphous alkaloid prepared by Schmidt, veratridine does not seem to have been employed in any pharmacological study. Crystalline cevine prepared by Freund and Schwarz was tested for its toxicity on frogs and rabbits by Falk (9), but only a very brief account of his studies was published.

The veratridine and the cevine used in our experiments were isolated by Professor R. P. Linstead[2] and Dr. D. Todd[2] from the batch of veratrine hydrochloride Merck used previously (1).[3] They also prepared the veratric acid and generously put at our disposal the following chemical data concerning the three substances. The amorphous veratridine, which represented between 40 and 50 per cent of the total alkaloids of veratrine hydrochloride Merck, was prepared according to Blount. It was regenerated from the crystalline sulfate and had a rotation of $[\alpha]^{31°}_{Hg} + 7.7°$ (2.5% in 95% ethanol). In confirmation

[1] This work was done under the auspices of the University Committee on Pharmacotherapy.

[2] Converse Laboratory, Department of Chemistry, Harvard University, Cambridge.

[3] We are indebted to Merck & Co. Inc., Rahway, N. J., for our supply of veratrine hydrochloride.

of what is stated in the chemical literature, the alkaloid had no sharp melting point; it shrank at 160 to 170° and decomposed at 170 to 178°. Cevine was isolated according to Macbeth and Robinson (10) and had a rotation of $[\alpha]_D^{26°}$ − 18.1° (2.4% in 95% ethanol). The veratric acid was a colorless crystalline substance and had a melting point of 180.5 to 181.5°C.

METHODS. Fifteen experiments on the denervated heart-lung preparation were conducted. The dogs used weighed from 7 6 to 14 kgm. They were anesthetized with nembutal, 35 mgm. per kgm. Defibrinated blood was used to supply the heart-lung preparation. The methods of recording changes in heart action are fully described in the first paper of this series. As in the earlier experiments, heart failure was either spontaneous or was induced by the injection of 0.05 to 0 225 grams of nembutal. The doses of veratridine or cevine mentioned below refer to the bases, although the alkaloids were injected in the form of the hydrochloride. The veratric acid was administered as sodium veratrate.

RESULTS. 1. *Veratridine.* In all respects the action of veratridine was qualitatively similar to that of veratrine. In the heart-lung preparation without signs of failure only a slight effect could be observed with non-toxic doses. When injected into the blood supply of the failing heart the symptoms of failure were reversed, as illustrated in figure 1.

Between the first and second segments of the tracing, 75 mgm. of sodium nembutal, added to the blood in the venous reservoir, caused a decrease in total output of 50% and a great increase in right auricular and pulmonary pressure. Under these conditions the heart was working at its maximum capacity, for raising the level of the venous reservoir 50 mm. produced only a negligible increase in output Within three minutes after the addition of 0 2 mgm. of veratridine to the blood in the reservoir, the auricular and pulmonary pressures fell to the initial value. Following the veratridine administration a 50 mm. elevation of the venous reservoir produced a 25% increase in the total output, with only a minor increase in auricular and pulmonary pressure. Occasional irregularities which had appeared with the nembutal failure ceased abruptly, but no significant change in the basic heart rate occurred other than the progressive slowing which is characteristic of the denervated heart-lung preparation.

Coronary flow in this experiment increased temporarily by 70% following the dose of veratridine and gradually diminished to the previous level within an hour. The effect on coronary flow is definite only when a state of failure exists prior to the veratridine administration and can rarely be repeated with subsequent doses. Similar effects upon cardiac output and auricular pressure occur when the failure is allowed to develop spontaneously (see fig. 2).

With effective doses of veratridine the diastolic volume of the heart decreases and the work of the heart always increases, but until studies of the cardiac oxygen consumption have been made it is not possible to say whether the veratrum alkaloids, like the cardiac glycosides, improve the mechanical efficiency of the heart.

The smallest effective dose of veratridine was about 0.05 mgm., at a concentration of 1:16,000,000. A maximal "therapeutic" effect, that is, a restoration of the normal output and normal auricular and pulmonary pressure, followed a dose of 0.2 to 0.3 mgm. In the average blood volume of these experiments, which was about 800 cc., this represented a concentration of approximately 1:4,000,000 to 1:2,500,000.

The duration as well as the magnitude of the veratridine effect upon cardiac dynamics is similar to that of veratrine; an initial dose of 0.2 mgm. produces an improvement in the failing heart which lasts for from 40 to 100 minutes, the maximum action occurring in about 5 minutes. Smaller doses have a shorter duration, and repeated doses produce a progressively smaller and briefer effect.

Repeated doses of veratridine eventually lead to arrhythmias which are interpreted as a toxic action of the alkaloid. When a toxic concentration has been reached, the heart rate may suddenly increase by 30 or 40%. The rate may remain quite regular, and the tachycardia may occur with asystole of the auri-

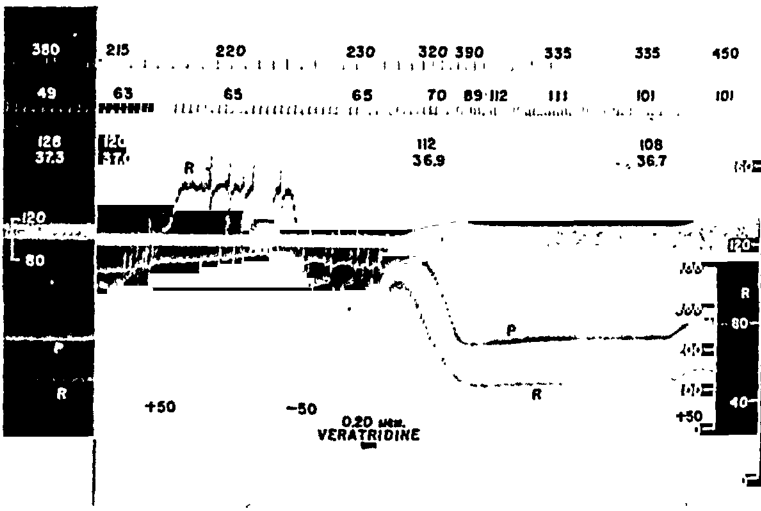


FIG. 1. Experiment 4. Action of veratridine upon the heart in nembutal failure. Heart-lung preparation. Dog weight 11.25 kgm. Ventricular weight 76 grams. Blood volume approximately 800 cc. Arterial resistance 80 mm. of mercury. Tracings from top to bottom: systemic output, each signal indicating 100 cc.; coronary sinus outflow, 8.5 cc. at each signal; time in minutes; arterial pressure (scale at left in mm. of mercury); pulmonary arterial pressure P (scale second from right in mm. of water); right auricular pressure R (scale at far right in mm. of water). The horizontal rows of figures indicate, from top to bottom: systemic output, cc. per minute; total coronary flow, cc. per minute; heart rate per minute; temperature of the blood in degrees centigrade; increase or decrease in the level of the venous reservoir in millimeters. Between the first and second segments, a time lapse of 50 minutes, 75 mgm. of sodium pentobarbital were injected.

cles, suggesting an A-V nodal or ventricular rhythm. Establishment of a permanent tachycardia is often preceded by short periods of acceleration of heart rate. Eventually toxic concentrations produce ventricular fibrillation of a slow type similar to that which frequently follows toxic doses of cardiac glycosides. In its toxic action veratridine is more potent than veratrine. Whereas as much as 1.5 mgm. of veratrine was injected within 80 minutes in one of the experiments previously reported without producing ventricular fibrillation, veratridine given in 3 doses of 0.2 mgm. each in a period of 50 minutes produced fatal irregularities in one experiment of the present series. In another experiment

the same effect was caused by several repeated doses totaling 0.75 mgm. given over a period of 90 minutes.

2. *Cevine.* Although much less potent than veratridine, the base cevine is not devoid of the characteristic activity. Initial doses of 20 mgm. or less have no definite therapeutic effect under conditions of heart failure in which 0.1 to 0.2 mgm. of veratridine have a marked action. In one of our experiments with nembutal failure 20 mgm. of cevine (27 mgm. per liter of blood) were inactive; in another experiment a dose of 20 mgm. (28 mgm. per liter of blood) produced a minimal response lasting for 15 minutes, comparable to the effects observed with 0.05 mgm. of veratridine.

In figure 2, 25 mgm. of cevine (33 mgm. per liter of blood, 27 mgm. per 100 grams of heart) were given to a heart with spontaneous failure. During $2\frac{1}{2}$ hours the heart volume had increased by 19 cc.; the administration of cevine was followed by a further increase of 6 cc., by an increase in right auricular pressure, and by a drop in systemic output. Subsequently 0.1 mgm. of veratridine produced a decrease of 19 cc. in the heart volume and increased the output of the heart. The effects produced by cevine given in a therapeutically effective initial dose are illustrated in figure 3. Failure was produced by 225 mgm. of nembutal added to the blood over a period of 40 minutes. At the time cevine was administered the systemic output had fallen by almost 50% from the initial value, and right auricular and pulmonary pressure had risen by 52 and 105 mm. of water respectively. Within 5 minutes after the administration of 70 mgm. of cevine, right auricular pressure had decreased by 35 mm. of water, pulmonary pressure by 85 mm., and the systemic output had increased to 90% of the initial level. The effect was long-lasting; failure of the same degree as that existing before the cevine was given, judged by right auricular pressure, did not occur until 100 minutes later. At that time 0.25 mgm. of veratridine was given (fig. 4). Within 2 minutes this drug produced an effect slightly greater than that caused by the large dose of cevine, and the improvement lasted for more than 90 minutes. Although coronary flow was not recorded in this experiment, it is safe to say that the total work of the heart was increased nearly 90% from the failure level by each of the alkaloids. Seventy mgm. of cevine produced a "therapeutic" response approximately equivalent to that of 0.2 mgm. of veratridine. In its "therapeutic" power cevine was therefore, per milligram, less than 1/300 as active as veratridine.

When an ineffective initial dose of 20 mgm. of cevine was followed within 20 to 40 minutes by subsequent doses, the second as well as further doses became effective. For instance, in one experiment in which heart volume was recorded (for actual time intervals between doses see bottom tracing of figure 5) four 20 mgm. doses were given to a heart in nembutal failure. The first dose produced no response; with the next there was a barely perceptible response; the third dose caused a significant decrease in heart volume lasting 30 minutes; and the fourth dose produced a similar although weaker effect lasting 27 minutes. A similar result was obtained in the experiment of figure 2 (for time intervals between doses see top tracing of figure 5). The initial dose of 25 mgm. of cevine

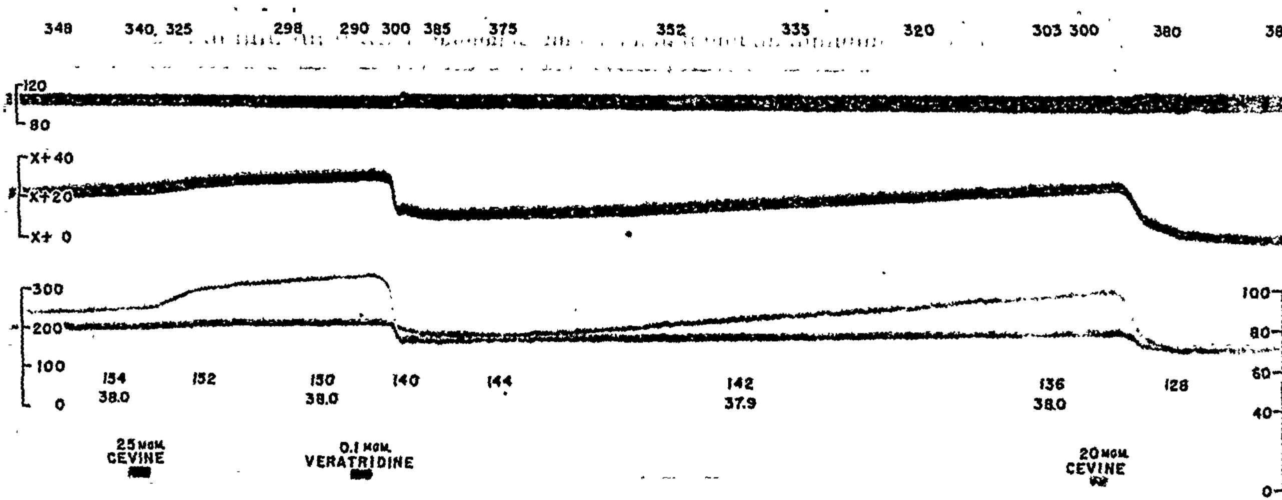
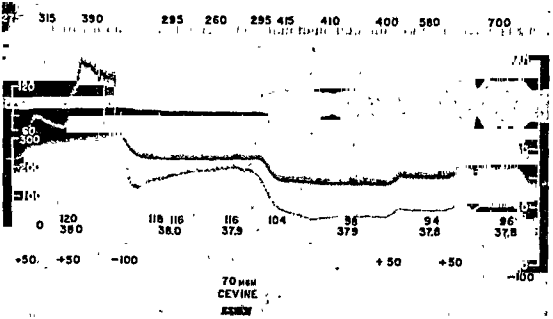


FIG. 2. Experiment 8. Action of cevine and veratridine upon dynamics and heart volume in spontaneous failure. Heart-lung preparation. Weight of dog 11.4 kgm. Weight of heart ventricles 92 grams. Blood volume approximately 800 cc. Arterial resistance 92 mm. of mercury. Tracings from top to bottom: systemic output, each signal representing 100 cc.; time in minutes; arterial pressure (scale at left in mm. of mercury); heart volume (scale at left in cc. above initial value); right auricular pressure (scale at right in mm. of water); pulmonary arterial pressure (scale at left in mm. of water). The horizontal rows of figures indicate, from top to bottom: systemic output in cc. per minute; heart rate per minute; temperature in degrees centigrade.

produced an impairment of cardiac work-capacity, as judged by the diastolic ventricular volume. When, after the effect of 0.1 mgm. of veratridine had worn off, failure had again progressed to the degree existing before the initial dose of



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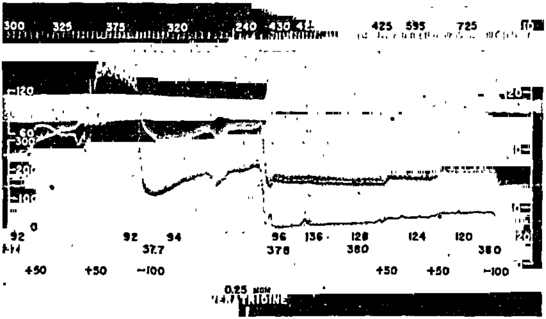

Fig. 4 Continuation of Experiment 6, legend as in figure 3. Time interval between figures 3 and 4, 85 minutes Blood volume at the time veratridine was given, approximately 610 cc

cevine, a second dose of cevine of 20 mgm. now produced a decrease of 26.5 cc. in diastolic volume and an increase of 27% in the systemic output, an effect which lasted 50 minutes. A further dose of 20 mgm. of cevine given when failure had again returned produced a response which was smaller and of shorter duration than that of the previous dose.

While veratridine in effective but sub-toxic doses does not produce any consistent rate effect, cevine in therapeutic doses causes a definite slowing of the heart rate (see fig. 5).

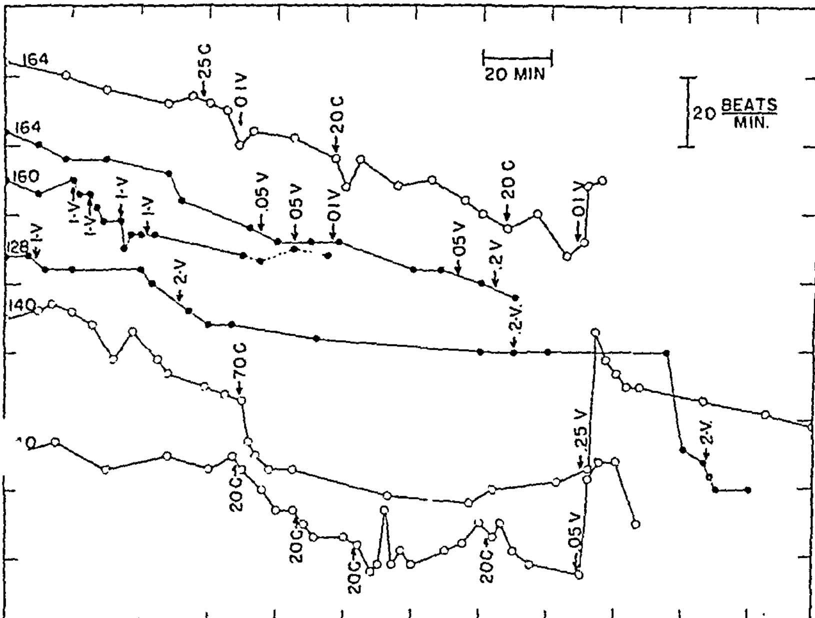

FIG. 5. Experiments 8, 3, 5, 4, 6, and 9 (from top to bottom). Effect of cevine and veratridine upon the rate of the denervated mammalian heart. Dogs. Heart-lung preparations. Each curve represents the rate changes in an individual experiment. The open circles designate experiments in which both cevine and veratridine were given; the solid points, experiments in which veratridine alone was administered. The figure at the beginning of each curve gives the initial heart rate in beats per minute. Doses indicated by the arrows are in milligrams. The broken line of Experiment 5 (third from top) represents the auricular rate during a period of 2:1 block. The abrupt decrease in heart rate at the end of Experiment 4 was caused by auricular asystole.

As toxic amounts are approached, periods of acceleration may appear. The marginal toxic dose of cevine was not accurately established, but a single dose of 70 mgm. corresponding to a concentration of approximately 1:10,000 failed to produce definite irregularities of rhythm. In one experiment marginal irregularities (short periods of acceleration) occurred after 80 mgm. of cevine had been given in four doses over a period of 75 minutes. Assuming no destruction, this would correspond to a concentration of 1:8,500. Similar marginal toxic effects

were observed in an experiment after one dose of veratridine of 0.1 mgm. and three doses of cevine totalling 65 mgm. Ventricular fibrillation did not occur in any of these experiments; the lethal concentration therefore cannot be stated.

In four experiments, three of which are shown in figure 5, veratridine in doses of 0.025, 0.05, 0.1 and 0.25 mgm., administered after previous doses of from 45 to 80 mgm. of cevine, caused an abrupt acceleration of the heart to rates nearly equal to the initial heart rate. This is evidence of an additive effect of the two drugs, for these doses of veratridine alone would have caused no definite rate effects.

3. *Veratric acid.* Veratric acid was administered in four experiments in doses up to 100 mgm. (1:9,000) in the normal heart, and up to 300 mgm. (1:2,500) in the failing heart. No effect could be obtained, and veratric acid did not appear to modify in any way the action of subsequent doses of veratridine or cevine.

Discussion. With regard to what we have described as therapeutic action upon the heart, the pure alkaloid veratridine is only slightly more potent than veratrine. The mixture of alkaloids exclusive of veratridine must therefore be almost as potent as veratridine; and a few experiments in which this mixture (its chief constituent being cevadine) was tested prove this assumption. The therapeutic potency of cevine is less than 1/300 of that of veratridine; and as the molecular weights of veratridine and cevine are 673 and 509 respectively, this relation of potency also holds on a molar basis. Falk, studying the toxicity in frogs and rabbits, observed that cevine was much less potent than cevadine; our experiments confirm this observation. It is the formation of an ester with a relatively simple, and in itself inactive, organic acid which augments so markedly the pharmacological potency of the base cevine. The effect of the combination of cevine with tiglic acid to form cevadine (9, 11) obviously is quite similar in this respect to the effect of the combination of cevine with veratric acid to form veratridine.

Judging by the therapeutic response of the isolated heart (decreased venous pressure and heart volume and increased output) repeated small doses of veratridine exert no cumulative effect. An ineffective dose (less than 0.05 mgm.) may be repeated at intervals of ten minutes for more than an hour with no evidence of improvement in the failing heart. Furthermore, the therapeutic response to an effective dose (0.05 to 0.2 mgm.) lasts for 15 to 60 minutes, after which a second dose produces a response of *lesser* magnitude and duration than before. After repeated doses, however, even though the therapeutic effect of each dose has worn off, a toxic effect of veratridine will appear, as evidenced by cardiac irregularities. The temporary nature of the recorded therapeutic response therefore is not proof of complete destruction or inactivation of the drug or of complete reversibility of the changes produced in the heart.

Repetition of ineffective initial doses of cevine (20 mgm.), unlike veratridine, eventually leads to a significant therapeutic response which wears off within 15 to 40 minutes. Further doses produce responses of lesser magnitude and duration and eventually result in disturbances of cardiac rhythm. It could be argued that the cevine activity is due to contamination with small amounts of a

highly potent alkaloid. In the case of a substance of a potency similar to that of veratridine, a contamination of 0.15% would mean 0.03 mgm. of the potent alkaloid for 20 mgm. of cevine—a second 20 mgm. dose therefore reaching the minimum effective dose. That the gradual development of the cevine action with repeated doses could be due to the gradual accumulation of an effective concentration of contaminating amounts of veratridine or of cevadine is excluded by the chemical procedure used in the preparation of cevine. Furthermore, such a contaminating alkaloid would have to have pharmacological qualities different from those of veratridine. This is suggested by experiments in which subminimal doses of veratridine were repeated at short intervals. In one experiment, for instance, spontaneous failure was allowed to develop within 90 minutes. Veratridine in doses of 0.04 mgm. was injected into the reservoir at intervals of ten minutes until a total of 0.2 mgm. had been given. Seven minutes later a dose of 0.075 mgm. was injected. There was no therapeutic response to any of these doses. Five minutes later 0.2 mgm. of veratridine produced a characteristic decrease in right auricular pressure and an increase in total output. In two other experiments the response to a small but effective dose of veratridine, 0.05 to 0.1 mgm., became smaller in magnitude and shorter in duration with repeated injections (for the time relation between successive doses see figure 5, second and third experiments from top). The most likely explanation of the increase in efficiency of repeated 20 mgm. doses of cevine is an accumulation of cevine in the blood, the therapeutic response occurring only after the threshold concentration has been reached. Apparently veratridine and cevine are additive in their therapeutic, and probably also toxic, actions on the heart.

SUMMARY

The cardiac action of the two pure veratrum alkaloids veratridine and cevine, and of veratric acid, was studied in the heart-lung preparation of the dog. Both alkaloids share the characteristic action upon the heart muscle which leads to an increase in the work of the heart, particularly in experimental heart failure. The effective and therapeutic doses are reported. Veratridine, which is an ester of cevine and veratric acid, is about 300 times more potent than cevine. Veratric acid is devoid of any characteristic action upon the mammalian heart.

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ACTION OF DIPHENYLOXAZOLIDINEDIONE ON BRAIN RESPIRATION AT VARIED TEMPERATURE LEVELS[1]

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While much information is available regarding the action of central nervous system depressants on brain metabolism *in vitro* (cf. 1), there are few instances in which sufficient data have been published to make concentration-action relations clear (2). This is partly due to the low solubility of many of the important depressants within the physiological pH range. Fewer still are the cases in which the effect of temperature upon these relations has been studied. In a previous paper (3) we described the effect of propazone (di-*n*-propyl oxazolidinedione) on the oxygen consumption of rat brain over a wide range of graded concentrations and at varied levels of temperature. In the present paper the action of another substituted oxazolidinedione, *5,5*-diphenyl-*2,4*-oxazolidinedione (hereinafter designated DPO) on the respiration of rat brain is reported. This drug was chosen for study because Stoughton and his associates (4, 5, 6), who first prepared the oxazolidinediones, reported that its action on the intact mouse is quite different from that of propazone in spite of the structural relationship.

METHODS. That the data on brain respiration in the literature are difficult to compare because of the variety of methods used has been pointed out by Elliott and Libet (7). Since standard procedures have been developed in this laboratory by which very constant rates of oxygen consumption are obtained with cerebral cortex slices for periods of three hours or more these procedures are described here, although various aspects have been published previously (3, 8).

1. *Preparation of tissue slices.* Twenty-three adult male albino rats were used. These were decapitated and the brain, stripped of meninges, was placed in a moist box maintained at 35°C., with a humidity of 100%. This took about two minutes. Slices of cerebral cortex were obtained by pressing a small lucite template down on the surface of the brain and shaving off with a razor blade the cortical tissue protruding through the slot in the template. Thin and quite uniform slices were prepared in this way. The slices were weighed immediately on a microtorsion balance and then either placed in crucibles for the determination of dry weight (the tissue being dried to constant weight in an electric oven at 105°C.) or in respirometer vessels. Using these procedures two workers can fill six respirometer vessels within ten minutes of the death of the animal. Features to be emphasized are that the tissues are not placed in suspension medium until after weighing, that dry weights are obtained on aliquots (not on samples fished out of respirometers after a run) and that the time between the death of the animal and the beginning of measurement of respiration is short and constant.

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M/100. Unless otherwise stated the medium contained 0.2% glucose. The gas phase was oxygen. Preliminary experiments showed that the oxygen consumption of cerebral cortex at 37.5°C. was higher in oxygen than in air, and was constant for more than three hours in oxygen whereas in air respiration diminished with time. The rate of shaking of the manometers was 100 complete oscillations per minute, with an arc of 8 cm. Thermostat temperature was 37.5°C. unless otherwise stated. Four thermostats were available, two of which were equipped with cooling units so that in the investigation of temperature effects simultaneous runs could be made on tissues from the same animal at four temperature levels, two of which could be below room temperature. Thus the effects of inter-individual variation were lessened. All temperatures were constant to ±0.02°C. Fifteen minutes of thermoequilibration preceded each run. Oxygen consumption was measured in cu.mm., N.P.T., per mgm. dry weight per hour (Q_{O_2}). When DPO was added, Q_{O_2} was calculated from readings taken 45 and 75 minutes after such addition at which time Q_{O_2} was constant. All pH measurements were made with a glass electrode.

3. *Preparation and use of diphenyloxazolidinedione.* This drug was kindly supplied by Dr. R. W. Stoughton. It was dissolved in weak sodium hydroxide and brought to pH 7.4. The resulting solution was diluted as desired. In all cases oxygen consumption was measured for a preliminary period of forty minutes before the drug was added from the sidearms of the vessels, so that each tissue sample furnished its own control. Each concentration of DPO was run in duplicate or in triplicate. Concentrations of DPO are given in mgm.% (i.e., mgm. per 100 cc. of solution) and also in gram-moles per liter when comparison with other data is easier on this basis (note: 100 mgm.% is equal to 3.95×10^{-3} M). Concentrations of propazone are given in mgm.% of the pure substance (not as propazone sodium), and in gram-moles per liter (note: 100 mgm.% propazone equals 5.41×10^{-3} M, 100 mgm.% propazone sodium equals 4.85×10^{-3} M). The corresponding molecular weights are DPO, 253; propazone, 185; propazone sodium, 207.

RESULTS AND DISCUSSION. 1. *Effect of DPO on the intact rat.* Stoughton and his associates have studied the effect of administration of substituted oxazolidinediones to intact mice. They reported that DPO was one of five of these compounds which produced convulsions without narcosis in the mouse; the other drugs of this series were central nervous system depressants (4, 5, 6). It is interesting that in a related series such as the barbiturates, while depression of the central nervous system is the characteristic action, some thirty-five convulsants have been reported (10 to 18).

To ascertain the effect of DPO on the rat, graded doses were administered by intravenous injection, using fluid volumes not exceeding 0.21 cc., to a small group of adult animals. We are indebted to Dr. J. M. Crismon of this laboratory for his kindness in doing this work. The mortality data are given in table 1.

In several cases the animals showed heightened excitability, but generalized symmetrical convulsions were not observed. On intraperitoneal injection of like doses in a few mice, heightened excitability was frequently observed; the animals would jump in a jerky fashion, with apparently synchronous and symmetrical contraction of all four legs when blown upon or when the adjoining surface of the table was struck. In both rats and mice central nervous system depression, evidenced by decreased excitability, did not develop in animals injected with DPO until just before death. In this respect there is a striking difference between DPO and propazone, since according to Stoughton *et al.* (6) propazone in proper dosage produces prolonged depression of the central nervous system, indicated by diminished response to sensory stimulation.

2. *Concentration-action relationships.* Rat cerebral cortex slices come into diffusion equilibrium with DPO quite rapidly. A level of Q_{O_2} which is characteristic and quite constant for at least two hours is attained not later than thirty minutes after addition of any given concentration of the drug. Such equilib-

TABLE 1

Effect of intravenous injection of DPO in rats

DOSE	NO. OF ANIMALS	LIVED	DIED
mgm. per kgm.			
20	5	5	0
40	4	3	1
70	4	1	3
100	3	0	3

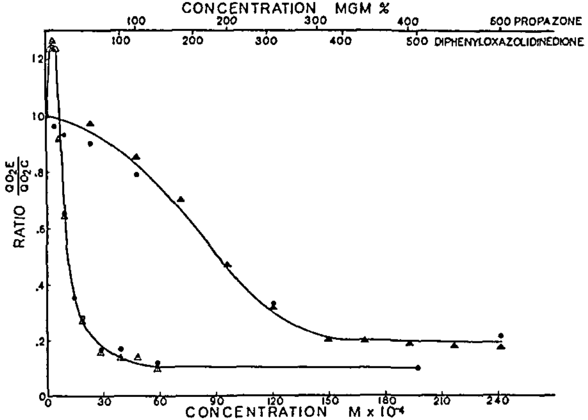

FIG. 1. CONCENTRATION ACTION CURVES SHOWING THE EFFECT OF GRADED CONCENTRATIONS OF DIPHENYLOXAZOLIDINEDIONE (DOTTED SYMBOLS) AND OF PROPAZONE (SOLID SYMBOLS) ON THE RESPIRATION OF RAT CEREBRAL CORTEX SLICES

rium levels of Q_{O_2} were used in the construction of the curves of figure 1, where the effect of graded concentrations of DPO on the oxygen consumption of cerebral cortex slices is shown. For comparison the concentration-action curve of propazone (the di-*n*-propyl homologue), calculated from data in an earlier paper (3) is also given in figure 1.

It is shown in figure 1 that as the concentration of DPO rises from subliminal levels there is first an augmentation, then an inhibition of brain respiration, while the augmentation phase is missing with propazone. It is also shown that the range between the initial inhibitory concentration and the concentration evoking maximum inhibition is much narrower in the case of DPO than of propazone. This rapid development of inhibition as the concentration of DPO rises from 8×10^{-4} M to 5×10^{-3} M is comparable to the rapid increase in inhibition of rat kidney respiration with rise in cyanide concentration from 10^{-4} to 10^{-3} M which van Heyningen described (19).

It is also shown in figure 1 that there is a small but definite fraction of cerebral cortex respiration which is not abolished even by very high concentrations of these substituted oxazolidinediones. This residual or inhibitor stable respiration is somewhat greater for propazone than for DPO. Similar residual fractions of respiration have been observed with other inhibitors such as dinitrophenol (20), cyanide (21), azide (22) and various barbiturates (22). Just what functional significance can be attached to inhibitor stable respiration is not yet clear.

3. *Substrate specificity.* Quastel and his associates (23, 24, 25) have shown that various "narcotics" including chloretone, ethyl urethane, scopolamine, a number of barbiturates and some other agents of like action exhibit considerable specificity in respect of their effects on brain respiration, the oxidation of glucose, lactate and pyruvate being inhibited at much lower drug concentrations than that of succinate or *p*-phenylenediamine. It was found that DPO resembles these agents in respect of its action on glucose and succinate oxidation. Thus in Ringer's-phosphate containing 0.011 M glucose, 75 mgm.% and 150 mgm.% DPO decreased the respiration of cerebral cortex slices to 17% and 12% of the control level respectively, while when 0.02 M sodium succinate was the substrate Q_{O_2} was much less reduced by these concentrations of DPO (to 84% and 73%, respectively, of the control level).

4. *Reversibility.* The reversibility of the inhibition of rat cerebral cortex respiration by propazone and by DPO in Ringer's-phosphate-glucose was tested by the method of Quastel and Wheatley (24). It is shown in table 2, Part A, that under the conditions of these experiments inhibition by propazone is in part reversible, while inhibition by DPO is not. The action of DPO was then tested for reversibility by the dilution method of Blaschko (26), using Machlett triple sac respirometer vessels (two sacs in Siamese form). It is shown in table 2, Part B, that inhibition of cerebral cortex respiration by the concentrations of DPO used was irreversible under these conditions also.

5. *Studies at graded temperatures.* The effect of temperature on augmentation and inhibition of rat cerebral cortex respiration by DPO is illustrated in figure 2. The concentrations of the drug tested were 10 mgm.% (which gives optimum augmentation at 37.5°C.) and 150 mgm.% which abolishes all but the inhibitor stable fraction of Q_{O_2} (cf. figure 1). A few additional comments on methods seem helpful here. The tissue slices in a given vessel were run at one temperature only. Other slices from the same brain were run concomitantly at

three other temperatures in the four thermostats available. Each point in figure 2 is calculated from mean data on three animals (run in duplicate for each animal) except only one animal was run at 20°C. with 10mgm.% DPO and only two at 30°C. with 10 mgm.% DPO.

It is shown in figure 2 that the diminution in "augmented" Q_{O_2} (curve A) and in control Q_{O_2} (curve B) with decrease in temperature is much more marked than in the case of the inhibitor-stable Q_{O_2} (curve C). Thus the inhibitor-stable Q_{O_2} is 10% of the control at 37.5°C. and 36% at 15°C. Qualitatively

TABLE 2

Reversibility of inhibition of rat cerebral cortex respiration by DPO and by propazone

Initial Q_{O_2} for first 10 to 40 minutes of run (control period). Experimental Q_{O_2} is Q_{O_2} for the period of 30 to 45 minutes after addition of drug or blank (Ringer's) from vessel sidearm. Q_{O_2} after washing or dilution represents Q_{O_2} after gentle washing in Ringer's (cf. 24) or after dilution of the drug by fresh Ringer's (cf. 26).

	INITIAL Q_{O_2}	EXPTL. Q_{O_2}	Q_{O_2} AFTER WASHING
Part A. Effect of washing—propazone and DPO			
Propazone			
Control (received blank)............	10.90	10.90	7.32
Propazone: 250 mgm.%............	11.40	4.84	5.15
Propazone: 500 mgm.%............	11.15	2.73	3.66
DPO			
Control............	11.20	11.20	7.73
DPO: 150 mgm.%............	9.95	2.26	1.13
Part B. Effect of dilution—DPO only			
			Q_{O_2} AFTER DILUTION
Control............	13.60	13.60	9.95
DPO: 25 mgm.%......	12.97	3.14	1.18*

* Final concentration of DPO after dilution, 2.5 mgm.%. Run in Siamese sidearm vessels. 0.2 cc. Ringer's with 50 mgm. tissue in one sidearm, 0.1 cc. DPO in Ringer's in the other. After "initial" period, DPO was added to sidearm containing tissue. In final period the total of 0.3 cc. in the sidearm was added to the 2 7 cc. of Ringer's in the vessel. Thus there was tenfold dilution.

similar findings have been described for cyanide (fertilized sea urchin eggs, 21) and for propazone (rat cerebral cortex, 3). Jowett's report (27) that various "narcotics" had less effect proportionately on rat brain respiration at 34°C. than at 39°C. is also in harmony with these findings.

Korr (21) has pointed out that when the ratio of control Q_{O_2} to experimental Q_{O_2} is plotted as a function of temperature, the relation between the temperature coefficients of the two processes may be inferred. Such a plot is shown in figure 3. Curves A and B represent values found when the denominator in the

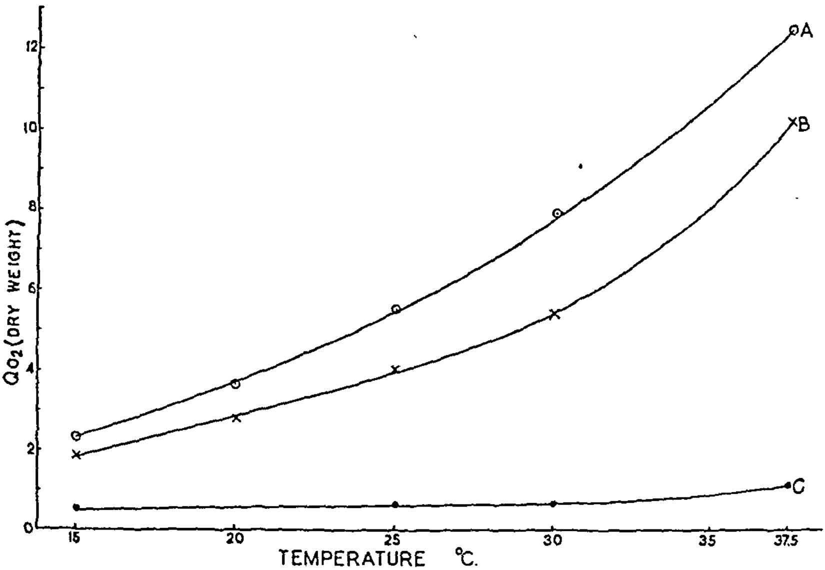

FIG. 2. AUGMENTED AND INHIBITED CEREBRAL CORTEX RESPIRATION AS A FUNCTION OF TEMPERATURE

Curve A: Qo_2 in presence of 10 mgm.% DPO (augmented). Curve B: Control. Curve Qo_2 in presence of 150 mgm.% DPO (inhibitor stable).

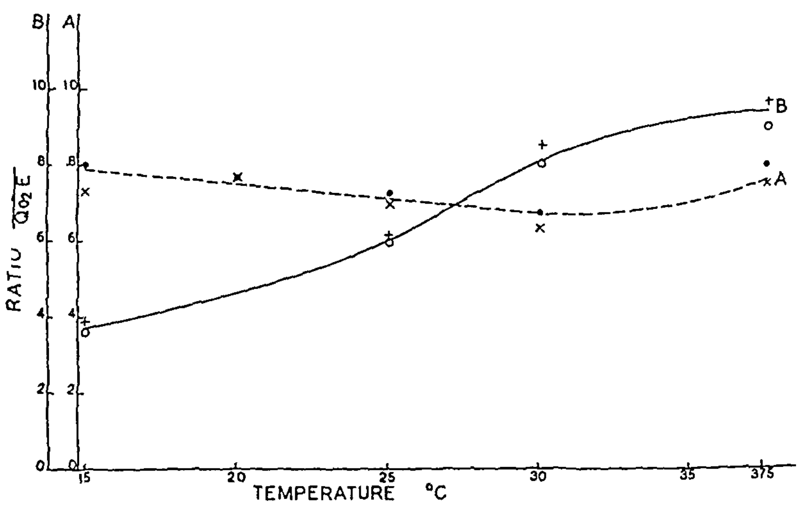

FIG. 3. THE RATIO OF Qo_2 C TO Qo_2 E (DEFINED AS IN LEGEND FOR FIG. 1) AS A FUNCTION OF TEMPERATURE

Curve A: DPO concentration 10 mgm.%. Curve B: DPO concentration 150 mgm.%

above ratio is Q_{O_2} in the presence of 10 and 150 mgm.%, respectively (Q_{O_2} E), of DPO (i.e., the levels of maximum augmentation and of inhibitorstable respiration) and the numerator is control Q_{O_2}. Since curve A is inclined downward with rising values of temperature to 30°C., it follows that the temperature coefficient (cf. Bélehrádek, 28) of the augmented respiration is greater than that of the control for the range 15° to 30°C. In the interval 30° to 37.5°C. these relations are reversed. The upward slope of curve B indicates that the temperature coefficient of the control respiration is greater than that of inhibitorstable respiration in this case. This is also shown by the properties of curve C, figure 2.

These experiments, like the analogous ones on propazone (3), serve to throw some light on the nature of the fraction of respiration called inhibitor-stable. In both instances this fraction is relatively stable toward cold also as indicated by figure 3, in which the picture is very like that obtained with propazone. Preliminary results indicate that this fraction is also characterized by a low R. Q.

SPECIAL DISCUSSION. The contribution made by these experiments to the theory of anesthesia is most easily discussed as a special topic. One of the more plausible hypotheses of the mechanism of narcosis (depression of normal functional activity of the central nervous system) is that of Quastel and his associates (1, 23, 25) that narcosis is consequent upon inhibition of the oxidation of glucose, pyruvate or lactate by brain cells. Since oxidation of glucose is the dominant energy-yielding reaction in the brain (1), one would expect disturbance in function if it were abolished or much diminished. However, the decrease in brain respiration *in vitro* produced by concentrations of narcotics evoking deep narcosis *in vivo* is not very impressive (1, 27). These observations led Quastel to suggest that the essential feature of narcosis might be local decrease in oxygen consumption in particular regions of the brain which has little effect on the oxygen consumption of brain as a whole (1).

An alternative explanation of the production of deep narcosis by concentrations of drugs having little effect on brain respiration *in vitro* is suggested but not proved by the data of this paper and by other data in the literature. Thus it has been shown that various drugs, structurally related to DPO, are concentrated in the brain *in vivo* to a level which, for a time at least, is several times that to be expected on the basis of uniform distribution (cf. 29, 30). Such relative concentration in the brain *in vivo* would reconcile our findings on the effect of graded doses of DPO *in vivo* (table 1) with our observations on the effect of graded concentrations on brain respiration *in vitro* (fig. 1), as shown by the following considerations. Injection of 20 mgm. per kg. of DPO (cf. table 1) would give a concentration of 2 mgm. % on the basis of uniform distribution. Three-fold concentration, (cf. 30), would raise this to 6 mgm.%, a level which would merely evoke a moderate rise in Q_{O_2} of excised brain slices. *A priori*, one would expect no deaths under these conditions and there were none. In contrast, threefold concentration of the 100 mgm. per kgm. dose would give a DPO level in the brain of 30 mgm.% (1.32×10^{-3} M), which would reduce Q_{O_2}

by about 60% (fig. 1). It seems likely that so profound a fall in brain respiration would lead to death (cf. 1, 31) and it is shown in Table I that administration of 100 mgm.% DPO to the intact rat resulted in the death of all three animals so treated.

These observations, together with the relevant findings in the literature, are in harmony with the view that the ureide narcotics and related compounds are concentrated in the brain *in vivo*. This conception would also account for the discrepancy between results *in vivo* and *in vitro* observed by Quastel and his co-workers (1, 27) without assuming unproved local effects.

While the hypothesis discussed above is in harmony with a number of observations in this and other laboratories, it is not the only reasonable explanation of our observations on DPO. Thus on the assumption of uniform distribution, concentrations of DPO causing only moderate rise in the Q_{O_2} of brain *in vitro* may bring about profound disturbance of some non-oxidative aspect of brain metabolism or function. Background for this hypothesis is afforded by the finding in this laboratory that *Triturus* toxin will kill rats in concentrations far below those affecting brain respiration or glycolysis *in vitro* (32) although there is evidence that the action of this toxin is central (33).

We are of opinion that the former hypothesis, that of relative concentration in the brain, is the more probable. Determination of the levels of DPO in the brain under the conditions of these experiments would supply crucial evidence. Unfortunately this would involve devising satisfactory quantitative methods for determining DPO in biological material, a task which we cannot undertake at present.

SUMMARY

1. Procedures were described whereby constant rates of oxygen consumption were obtained with cerebral cortex slices for periods exceeding three hours.

2. The effect of intravenous injection of graded doses of *5,5*-diphenyl-*2,4*-oxazolidinedione (DPO) in minimal volume, to a small group of rats was reported for the dosage range of 20 to 100 mgm. per kgm.

3. A concentration-action curve was presented which illustrates the effect of graded concentrations of DPO on the oxygen consumption of rat cerebral cortex slices. This was compared with the concentration-action curve of the related substituted oxazolidinedione, propazone. With rising concentration of DPO there was first a moderate augmentation, then a profound inhibition of brain respiration. The augmentation phase did not occur with propazone. Furthermore there was more rapid development of inhibition and a greater maximum inhibitory effect with DPO than with propazone.

4. It was found that DPO had some specificity in respect of its inhibitory action on brain respiration. Like propazone and like various narcotics, DPO decreased the oxygen consumption of cerebral cortex slices much more markedly in the presence of glucose than in the presence of succinate.

5. Under the conditions of these experiments the inhibition of brain respira-

tion by DPO was not reversible, whereas the inhibition by propazone was in large part reversible.

6. Inhibition of brain respiration by DPO became relatively less marked with decrease in temperature. Thus at 37.5°C. only 10% of the respiration was stable toward DPO, while at 15°C. this fraction was 36%.

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TOXICITY OF ROTENONE AND DERRIS EXTRACT ADMINISTERED ORALLY TO BIRDS[1]

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The use of rotenone, the principal toxic constituent of derris, and of derris root itself in insecticidal operations, has assumed considerable importance in economic entomology. This is chiefly due to the marked toxicity to insects and the comparative safety to humans. The toxicity of these materials to higher animals which might be subjected to their action has been determined for several different mammals. It is important to know just how toxic these materials could be to birds which might encounter rotenone or derris sprayed or dusted on insects which are injuring crops.

The most susceptible mammal tested appears to be the guinea pig. The values of approximate median lethal doses of pure rotenone administered orally to this species range from 0.050 (1, 2), to 0.100 to 0.200 milligrams of toxic material per gram of body weight as determined by Shimkin and Anderson (3). Rabbits appear most resistant, the minimum lethal dose being 3.0 mgm. per gram of body weight (2). In experiments involving birds Haag (4) indicated that the median lethal dose for pigeons would be greater than 0.5 mgm. per gram, and Whittaker and Whittaker (5) fed two 0.02 gram doses to several four-week old chicken cockerels without harmful effects.

This study was undertaken to determine the oral toxicity of rotenone to several species of birds. Derris extract and derris root were also used for comparative purposes.

Materials and Methods. The following materials were used in this study:

Chemically pure rotenone. One sample used during 1940 and 1941 supplied by S. B. Penick and Co. Second sample received fresh from John Powell and Co., Inc. in 1942 and used during that year. Particle size of both samples averaged about 30 microns. At least 85% of samples would pass 325 mesh.

Derris extract. Sample supplied by John Powell and Co., Inc. Contained 25% rotenone, known commercially as derris resinate. About 85% of particles would pass 325 mesh screen.

Derris dust. Finely ground 5% derris root dust supplied by John Powell and Co., Inc. and diluted with Eastern Magnesium Talc to a dust with 0.75% rotenone content.

Most of the oral administration of rotenone was accomplished by feeding birds gelatin capsules containing amounts of rotenone or derris extract weighed accurately to 0.1 mgm. Empty capsules and capsules containing sublethal doses constituted controls. All nestling birds were treated in the wild state at the location where found. Effort was made to keep all conditions, except for rotenone administration, as nearly normal as possible. Nestling birds were removed from their nests, weighed in grams, fed toxic material and returned to the nest. Markings were by celluloid bands on legs and white thread loosely tied about humerus of wing. Observations on nestlings were carried out 12 to 24 hours after rotenone

[1] Direction and advice given by Dr. Roy Hansberry is gratefully acknowledged; thanks are also due Dr. A. A. Allen for numerous suggestions.

administration. Surviving birds were examined at intervals until birds left the nest or appeared normal. Difficulties in observations were encountered when parent birds removed dead young from the nest. Nestlings which could not be accounted for because of this or because of nest desertion, or difficulties which did not appear due to rotenone were not considered in determination of median lethal dosages.

Chickens, pheasants, and adult birds of English Sparrows and Prairie Horned Larks were fed adequately and housed under conditions that would approach normal. Adult birds were allowed to fly about in a portion of a greenhouse.

Rotenone was also administered by thoroughly mixing in starting-growing mash of the pheasant diet, and allowing the pheasants to eat various doses. Derris dust was administered to nestling Eastern Robins by rolling suitable insect larvae in the dust to obtain a maximum deposit, then feeding dusted larvae to the nestling until the bird refused additional ones.

RESULTS. The oral toxicity of chemically pure rotenone was determined approximately for twelve different species of nestling birds by administration of toxic material in gelatin capsules.

Results of the administrations are presented in tables 1 and 2, and figure 1. The Eastern Chipping Sparrow appeared most susceptible, the median lethal dose (MLD) being estimated at 0.113 mgm. per gram based upon individuals in the intermediate zone, which includes values from the smallest fatal dose to the largest surviving dose (6). Eastern Robins and English Sparrows were tested most extensively and their MLD's were quite similar. The values of 0.195 and 0.199 mgm. per gram, respectively, were determined by Bliss' method (7) for determining dosage-mortality curves for small numbers. The Eastern Yellow Warbler appeared to be the most resistant nestling bird tested, but insufficient experimental individuals prevent an accurate estimation of the MLD. The value appears to lie between 0.361 and 1.47 mgm. per gram.

Different responses to rotenone were studied with different age groups within a species. Five-day old chickens of mixed breeds had an MLD of 0.996 mgm. per gram (table 2), in contrast to the 3.077 mgm. per gram value which was determined as the MLD for four-week old chickens of Leghorn stock. The older group appeared more than three times as resistant to rotenone. The difference between pheasants of different ages was not so striking, but the 30-day group had an MLD of approximately 1.2 mgm. per gram as compared to the lower value of 0.85, the approximate MLD for five-day old pheasants. More conclusive results of age differences in susceptibility were revealed in tests with English Sparrows. Nestlings gave an MLD of 0.199 mgm. per gram by Bliss' method of calculation, and 12 immature birds, an arbitrarily selected group hatched during the season of experimentation and weighing over 23 grams, gave an approximate MLD of 0.4 mgm. per gram as contrasted to 0.853 mgm. per gram, the MLD for adults. In addition, 5 adult Prairie Horned Larks were fed doses shown in table 3, the MLD being estimated at 0.44 to 0.50 mgm. per gram. This value is above the probable MLD for nestling Prairie Horned Larks. The latter value appeared to be <0.23 mgm. per gram.

Derris extract containing 25% rotenone was compared directly with chemi-

TABLE 1

Responses of eight species of nestling birds of about 8 to 10 days old to chemically pure rotenone ingested by mouth in a gelatin capsule. Each dosage is listed in milligrams of rotenone per gram of body weight and applies to one individual

SPECIES	LETHAL ZONE DIED	INTERMEDIATE ZONE Died	INTERMEDIATE ZONE Survived	SUBLETHAL ZONE SURVIVED
Estimated MLD 0.361 to 1.47 mgm. per gram				
Eastern Yellow Warbler, *Dendroica aestiva aestiva* (Gmelin)	1.47 1.47 10.00			0.073 0.110 0.167 0.310 0.361
Estimated MLD <0.129 mgm. per gram				
Eastern Meadowlark, *Sturnella magna magna* (Linnaeus)	0.129 0.178 0.275 0.355			
Estimated MLD <0.2 mgm. per gram				
Cedar Waxwing, *Bombycilla cedrorum* Vieillot	0.2			
Estimated MLD <0.23 mgm. per gram				
Prairie Horned Lark, *Otocoris alpestris praticola* Henshaw	0.23			
Estimated MLD <0.322 mgm. per gram				
Least Flycatcher, *Empidonax minimus* (W. M. & S. F. Baird)	0.322 0.333			
Estimated MLD <0.38 mgm. per gram				
Eastern Cowbird, *Molothrus ater ater* (Boddaert)	0.38			
Estimated MLD <0.414 mgm. per gram				
Eastern Mourning Dove, *Zenaidura macroura carolinensis* (Linnaeus)	0.414 0.097*	(gone from nest, record uncertain)		
Estimated MLD 0.1 to 0.145 mgm. per gram				
Pigeon, *Columba livia livia* Gmelin	0.145 0.178 0.526			0.098

cally pure rotenone using five-day old chickens of mixed breeds. Table 2 shows the wide difference, 0.247 and 0.996 mgm. per gram, respectively. Thus an equivalent amount of derris extract was slightly more toxic than chemically pure rotenone.

A derris dust containing 0.75% rotenone was coated on various insect larvae and fed to nestling Eastern Robins. The amount of dust deposit per larva varied considerably with different species. The smaller cankerworms and ugly-

TABLE 2

Responses of different species of birds to chemically pure rotenone ingested by mouth in a gelatin capsule. These species were tested in numbers sufficient to give rather accurate determinations of median lethal dose (MLD) expressed in milligrams of rotenone per gram of body weight

SPECIES	NUMBER OF INDIVIDUALS	AGE	NUMBER IN INTERMEDIATE ZONE	MLD	VARIATION*
		days		*mgm. per gram*	
Eastern Chipping Sparrow, *Spizella passerina passerina* Bechstein	16	3–10	10	0.113	
Eastern Song Sparrow, *Melospiza melodia melodia* (Wilson)	13	3–10	8	0.130	
Eastern Robin, *Turdus migratorius migratorius* Linnaeus	46	3–10	26	0.195	0.094 to 0.407
English Sparrow, *Passer domesticus domesticus* (Linnaeus)	37	3–10	25	0.199	0.185 to 0.214
Chickens (using derris extract, containing 25% rotenone)	27	5	14	0 247	0.166 to 0.366
English Sparrow	14	Adult	7	0.853	
Pheasant, *Phasianus colchicus torquatus* (Gmelin)	15	5	12	0.85	
Chickens	52	5	28	0.996	0.563 to 1.747
Pheasants	8	30	6	1.19	
Pheasants (mixed in feed)	8	30	6	1.25	
Chickens	116	28	31	3.077	

* Computed limits of doses which would occur in 19 cases out of 20, or in 95% of the cases.

nest caterpillars retained approximately 0.56 mgm. per larva as compared to 7.1 for silkworms and 9.2 for imported cabbage worms. Results given in table 4 show that as few as eleven heavily-coated imported cabbage worm larvae could prove fatal to nestling birds. The possibility exists that larvae may contribute some toxicity themselves. Survivors shown in table 4 indicate that no toxicity was evident, and in addition a number of nestling Robins and Eastern Goldfinches have been fed to capacity on larvae coated with relatively inert walnut dust without any outward symptoms. The lowest toxic value of rotenone in

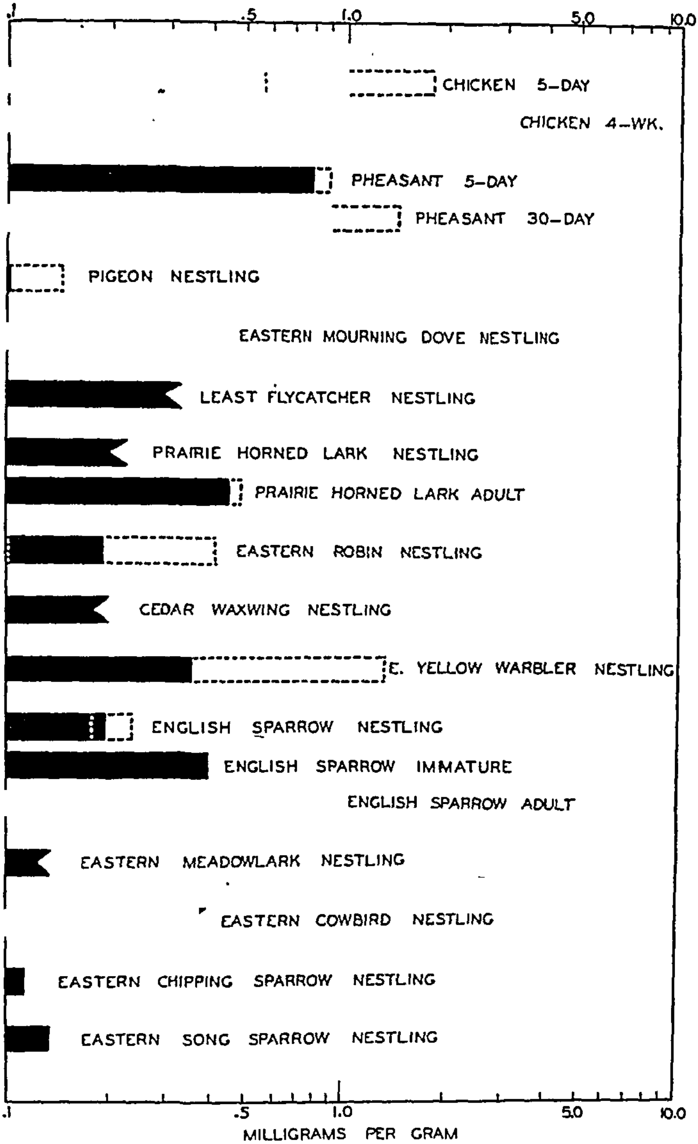

FIG. 1. ORAL TOXICITY OF CHEMICALLY PURE ROTENONE TO VARIOUS SPECIES OF BIRDS

Correct MLD somewhat less than maximum value indicated.

MLD at end of solid black portion. Variation, or limits of doses in 19 cases out of 20 indicated by dotted portions.

Extremes to which MLD may extend.

derris dust was 0.008 mgm. per gram. This value is approximately one-twenty-fifth the value of 0.195 which was obtained with chemically pure rotenone.

TABLE 3

*Response of 5 adult Prairie Horned Larks, Otocoris alpestris praticola Henshaw to chemically pure rotenone ingested by mouth in a gelatin capsule. Each dosage is listed in milligrams of rotenone per gram of body weight and applies to one administration**

LETHAL ZONE DIED	INTERMEDIATE ZONE		SUBLETHAL ZONE SURVIVED
	Died	Survived	
0.894 (E)	0.442 (A)	0.448 (B)	0.022 (A)
	0.479 (D)	0.498 (D)	0.053 (B)
	0.493 (C)		0.138 (C)
			0.433 (B)

Estimated MLD 0.45 to 0.50 mgm. per gram.

* Five adults were involved in this experiment; they are designated as A, B, C, D, and E. Thus, it can be seen that B was fed rotenone 3 times and survived all feedings. The doses were not spaced closer than at two week intervals in which time the bird appeared to return to normal activities, regaining any weight which may have been lost due to the sublethal dose.

TABLE 4

Results of administration of various insect larvae coated with a derris dust containing 0.75% rotenone to nestling Eastern Robins of 8 to 10 day age

SPECIES INGESTED	NUMBER OF LARVAE	MGM. OF DUST	MGM. ROTENONE	MGM. PER GRAM	RESULT
Ugly-nest caterpillar, *Archips cerasivorana* (Fitch)	24	13.4	0.101	0.003	Alive
	25	14.0	0.105	0.005	Alive
Fall cankerworm, *Alsophila pometaria* (Harr.)	71	39 8	0.298	0.008	Dead
	90	50.4	0.378	0.008	Dead
Imported cabbage worm, *Pieris rapae* (L.)	7	64.4	0.483	0.012	Alive
	8	73.6	0.552	0.015	Alive
	11	101.2	0.759	0.025	Dead
Silkworm, *Bombyx mori* (L.)	17	120.7	0.905	0.030	Dead
	18	127.8	0.949	0.034	Dead

DISCUSSION. The oral administration of chemically pure rotenone to 12 different nestling birds gave a range of doses, expressed as MLD's, from approximately 0.1 to 0.3 mgm. per gram. The Eastern Yellow Warbler was an exception, the MLD appearing to be greater than 0.361, although an intermediate zone was not determined for this species. The experimental results indicate that nestling birds are about as susceptible to rotenone as are guinea pigs. Species differences among nestling birds did not appear great.

Wide differences in response were shown by old and young birds of the same

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THE RELATION OF MOLECULAR CONFIGURATION TO INACTIVATION OF SYMPATHOMIMETIC AMINES IN THE PRESENCE OF PHENOL OXIDASE

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Since 1907 when Bertrand described tryaminase (1) it has been appreciated that this enzyme activates the oxidation of aromatic compounds having an hydroxyl group in the *para* position on the ring. In 1941 we published some general findings (2) on the enzymic oxidation of substituted phenylpropylamines by amine oxidase (aminase) and phenol oxidase (phenolase, tyrosinase, tyraminase). Until then it had not been recognized that the molecular configuration of the side chain of these phenolic sympathomimetic amines could materially influence the rate of oxidation of the nuclear structure.

It has been the purpose of the present work to study extensively the relation of chemical structure of phenolic pressor amines to their rate of oxidative inactivation in the presence of phenolase. Results obtainable from such an undertaking might possibly give a physical-chemical basis for (*1*) an interpretation of enzyme specificity in this particular instance, (*2*) the induced effect of substitution in a distant part of a molecule on the rate of a definite reaction pattern. Whether or not inactivation of these compounds in the mammalian organism by a phenolase occurs these intra molecular influences of various groups might be expected to alter similarly any *in vivo* system activating the oxidation of the phenolic structure.

PROCEDURE. We previously described the preparation of the phenolase extract from potatoes and the general procedure used in this research (2). From a practical point of view the crude enzyme preparation used in these experiments is sufficiently concentrated to facilitate rapid oxidation of moderate amounts of the amines (0.2 cc., M/16), is easily prepared and is relatively stable for at least 72 hours when kept in the refrigerator. The preparation has a definite advantage in that it retains the components of this enzyme system necessary for the immediate activation of the oxidation of monohydroxy phenyl derivatives.

The oxygen taken up in the course of the reactions was measured by means of the Warburg respirometer, the flasks being shaken at a constant temperature of 38°C. The flasks contained:

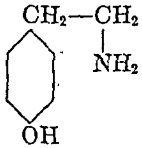
1.5 cc. phenol oxidase preparation
0 3 cc. M/4 Na-K phosphate buffer, pH 7 0
0.2 cc. M/16 amine (in terms of the organic ion), or H_2O as controls.

2 0 cc. total volume.

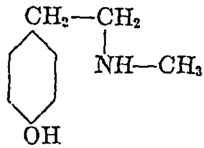
Duplicate determinations were done in each experiment. At least 4 and usually 6 or 8 experiments were performed with each compound. Always a reference compound was included among the 5 amines tested at a given time. The reference compound was tyramine for the phenylethylamine derivatives, paredrine for the phenylisopropylamines. This was done as a further check on the relative rates of reaction of the different compounds.

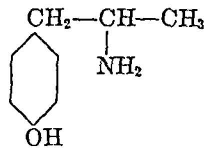
In all the experiments filter paper saturated with 10% KOH was contained in the wells of the flasks as a precautionary measure to adsorb CO_2 evolved in the course of the experiment

RESULTS. *Oxidation of para hydroxyphenylethylamines.* The amines used are represented by the following structural formulas:

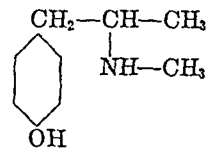


Tyramine





Paredrine

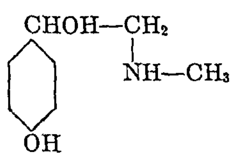


Paredrinol

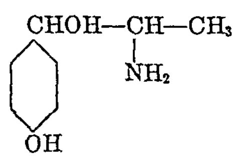
These compounds were all rapidly oxidized in the presence of phenolase as is shown in figures 1 and 2, curves 1 and 2. The length of the side chain did not influence the rate of oxidation nor did the presence of a secondary methyl ımino group alter the reaction.

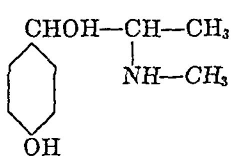
In this work we did not carry the reactions to completion in every instance. his was done previously (2) for the compounds studied at that time. The urpose of that work was to consider the atomic equivalents of oxygen taken up n the course of the reaction, to propose the steps in the over all reaction and to how that in the examples cited the total O_2 uptake was independent of the ate of the reaction.

Influence of aliphatic hydroxyl and ketone groups on the rate of oxidation of he *para* phenolic nucleus. The compounds used were:



Synephrin





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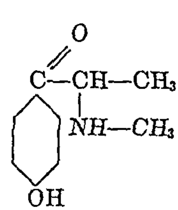


Figure 1 curve 4 and figure 2 curves 3 and 4 represent the effect of an aliphatic hydroxyl group on the carbon atom adjacent to the ring on the rate of the reactions involving the *p*-phenolic compounds. From these curves it may be seen that invariably the aliphatic hydroxyl group decreased the rate of the initial reaction involving phenolase. For the two compounds having the isopropyl side chain (fig. 2) the degree of the retarding effect is practically identical

irrespective of the primary or secondary amino group. The decreased rate of oxidation in the case of synephrin, figure 1 curve 4, is of the same order as that of the other two compounds, which indicates that the aliphatic hydroxyl group is responsible for this inhibitory phenomenon.

We observed this same inhibitory effect in the instance of conjugation reactions of diazonium compounds with sympathomimetic amines (3). Preferentially, if both a *p*-hydroxyl and a primary aliphatic amino group occurred in the same compound, coupling with a diazonium reagent occurred through and adjacent to the OH group. However, in the instance of *p*-hydroxyphenyl-β-hydroxyisopropylamine coupling with *p*-nitrobenzenediazonium chloride oc-

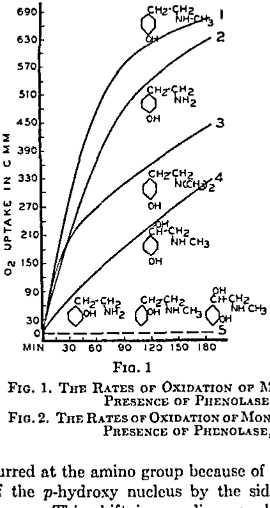


Fig. 1

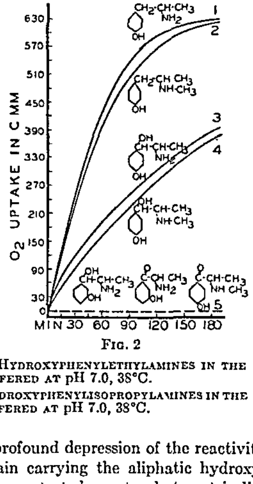


Fig. 2

Fig. 1. The Rates of Oxidation of Mono-Hydroxyphenylethylamines in the Presence of Phenolase, Buffered at pH 7.0, 38°C.

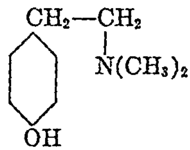
Fig. 2. The Rates of Oxidation of Mono-Hydroxyphenylisopropylamines in the Presence of Phenolase, Buffered at pH 7.0, 38°C.

curred at the amino group because of the profound depression of the reactivity of the *p*-hydroxy nucleus by the side chain carrying the aliphatic hydroxyl group. This shift in coupling can be demonstrated spectrophotometrically.

Since the aliphatic hydroxyl group so markedly decreased the rate of initial oxidation of the *p*-phenolic ring in the presence of phenolase it was decided to investigate this phenomenon further. Whereas the aliphatic hydroxyl group has a sign and approximate dipole moment of -1.83×10^{-18}, the values for the corresponding ketone are -2.79×10^{-18} (4). Because of the similar sign and greater magnitude of the dipole moment of the ketone group it was reasoned that its presence should reduce the reactivity of the *p*-phenolic ring to a greater extent than did the aliphatic hydroxyl group. We studied the effect of phenolase

on the ketone of suprifen and found that the compound completely resisted oxidation in the presence of this enzyme (fig. 2 curve 5).

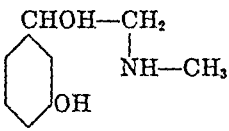
A tertiary amino group on the side chain as in hordenine was associated with a



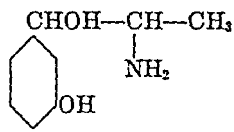
Hordenine

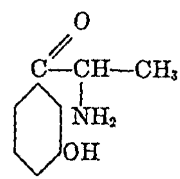
slower rate of oxidation of the ring than occurred in the case of its homologs having a primary or secondary amino group on the side chain, figure 1 curve 3.

Meta hydroxy phenylethylamines were not oxidized in the presence of phenolase, figures 1 and 2 curve 5. The compounds studied were:



Neosynephrin

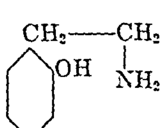


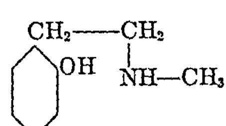


The experimental conditions were the same as for the previously studied compounds.

From the three compounds available to us we were not able to determine definitely whether the aromatic m-OH or the aliphatic OH group was primarily responsible for this refractoriness. Since the aliphatic hydroxyl radical only retarded the rate of oxidation of the p-OH homologs we felt that the m-OH group was the determining factor. This was substantiated by a recent report by Alles, Blohm and Saunders (5) in which they observed that m-hydroxyphenylethylamine was not oxidized in the presence of phenolase. Our observations lead us to obtain o-hydroxy homologs for a systematic study of the enzyme specificity of phenolase.

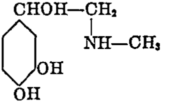
Ortho hydroxy phenylethylamines, like the m-hydroxy compounds, were not oxidized in the presence of phenolase. The compounds used were:



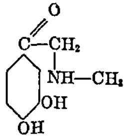


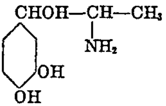
An explanation for these observations concerning o and m derivatives is presented in the interpretation of the results.

Oxidation of ortho dihydroxyphenolic sympathomimetic amines. The compounds tested were:

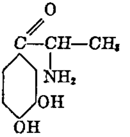


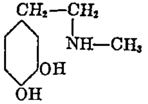
Adrenalin



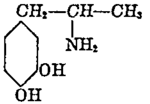


Cobefrin





Epinine



This group of compounds enabled us to investigate other aspects of the problem of the relation of structure to rate of oxidation. From figure 3 it can be calculated that until about two and one half atoms of O_2 were taken up in the course of the oxidation the rate of reaction for all these compounds was similar.

Referring back to the *p*-hydroxy agents it will be remembered that an hydroxyl group on the side chain next to the ring decreased the rate of oxidation of the nucleus whereas the corresponding ketone group completely inhibited reactivity of the ring. Comparing the rate of oxidation of adrenalin and its ketone with the corresponding *p*-hydroxy homolog synephrin, or cobefrin and its ketone with the *p*-hydroxyphenyl compounds suprifen and its ketone it must be concluded that: (*1*) the inhibitory effect of these side chain substituents was much greater on the initial reactivity of the *p*-phenolic nucleus. (*2*) The rate of oxidation of the *o*-dihydroxy nucleus formed as the first step in the oxidation of the *p*-hydroxy compounds was dependent primarily on its rate of formation. Substantiating this second point are the curves in figure 3 which show the oxidation of these compounds to be unimolecular in nature and the rate to be dependent on the concentration of the substrate at a given time.

It should be noted that in figure 3 the curves for the compounds tended to cross within a range of about 10 cu.mm. and about the same time. This crossing occurred at an oxygen uptake which was almost exactly two and one half atoms of oxygen per molecule of substrate. One wonders whether the observation is significant. At the termination of the experiments graphed in figure 3 the oxygen uptake for the group as a whole was within +3.5 and −8.7% of the 425 cu.mm. approximately equivalent to 3 atoms of O_2 per molecule of substrate which we previously found to hold for this group of compounds (2).

INTERPRETATION. It can be shown that the oxidation of typical compounds of this series, at least through the *o*-quinone stage, is a reaction of the first order, figure 4. The velocity of organic reactions such as these is unimolecular in character since they depend on the speed of activation of only one organic reagent (6). The equation for such a reaction velocity has been written

$$k = PSZ_e^{-E/RT}$$

where k is the reaction velocity, Z is the collision frequency of the reactants, P and S are probability terms depending primarily on steric conditions of the

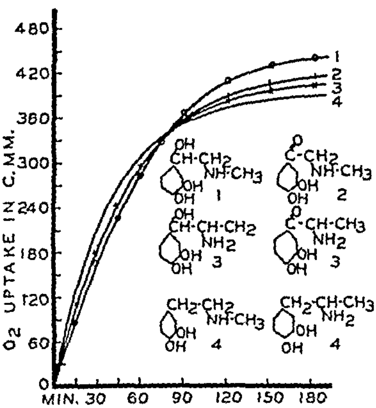


FIG. 3

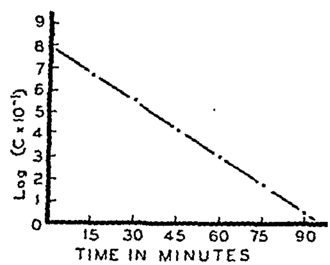


FIG. 4

FIG. 3. THE RATES OF OXIDATION OF *o*-DIHYDROXYPHENOLIC SYMPATHOMIMETIC AMINES IN THE PRESENCE OF PHENOLASE, BUFFERED AT pH 7.0, 38°C.

FIG. 4. For a reaction of the first order the Log of the concentration of substrate is a linear function of time. This curve is constructed from data presented in figure 3 curve 3 for cobefrin and is representative of the group. The values for Log C were derived as follows. The initial concentration of substrate was 6.25×10^{-2} mols. The volume of O_2 necessary to bring about the complete oxidation of this substrate was 425 cu.mm. (2). Then at any time T, $C = O_2$ uptake/$425 \times (6.25 \times 10^{-2})$. Using the data presented in figure 6 (Beyer (2)) it can be shown that the coordinates of the Log C/T curves for paredrine and cobefrin are practically identical, as they should be under optimal conditions. While the coordinates for this curve were calculated on the basis of 425 cu.mm. for 3 atoms of O_2 taken up in the over-all reaction (690 cu.mm. O_2 equivalent to 5 atoms O_2/mol. of paredrine) we recognize that the rate of uptake of the last atom of oxygen, which was not catalyzed directly by phenolase, was not necessarily of this same order. However, calculating the coordinates of this curve for the rate of the reaction involving an uptake of only 2 atoms of O_2 for the catechol derivatives or 4 atoms of O_2 for the oxidation of paredrine, attributable to the presence of phenolase, would only change the slope and not the character of the curve.

reactants. Bradford and Jones (7) working on the chlorination of anilides and Williams and Hinshelwood (8) studying the velocity of benzoylation of amines concluded that the factors P and S had little to do with differences in velocity of a given reaction, which differences they attributed primarily to E, the energy of activation. The above equation, then, may be written

$$k = Be^{-E/RT}$$

for reactions between covalent bonds and ions, since B is nearly equal to the frequency of collision of the reactants and is relatively independent of their structure. According to Williams and Hinshelwood marked electronic displacements caused by substitutions into a molecule may change E considerably while having far less effect on B. In effect, then, in our experiments B, R and T remain relatively constant and k varies inversely with E since its sign is negative.

We have preferred to interpret the relation of the enzyme to the substrate in terms of the quantum theory of Medwedew (9) for enzymic reactions. In accordance with this theory, the enzyme in order to activate the oxidation of the substrate must become activated through taking on a quantum of energy $h\gamma$. This energy is derived from the breakdown of an *o*-dihydroxy phenolic component of the enzyme system from the potato.[1] Through the collision of the activated enzyme with a molecule of the substrate this quantum of energy is transferred to the substrate. If this added quantum of energy $h\gamma$ is greater than the energy of activation E, it will bring about the characteristic orbital rearrangements of the reactant molecules which is the reaction. The energy for reactivation of the enzyme comes from the reactions involving later steps in the oxidation of the substrate.

The quantum of energy the enzyme is capable of transmitting to the substrate for initiating the reaction would remain constant and independent of the position of the hydroxyl group on the ring of the substrate or of substitutions into its side chain. But the component of E attributable to the substrate can be resolved into two parts: (*1*) a constant Eo representing the intrinsic energy of activation, under given experimental conditions, of the bond which is being acted on and (*2*) a variable ΣEp representing variations induced in the polarization of the molecule by substituent or distant atoms. Thus

$$E = Eo + \Sigma Ep$$

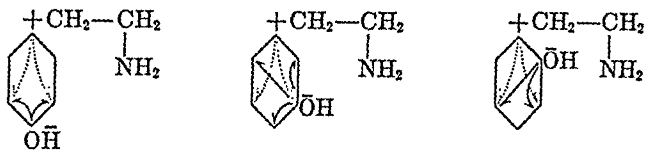
The factor ΣEp in these compounds was determined by the position of the phenolic group with respect to the side chain or the substitution of hydroxyl or ketone groups into the side chain since these relationships influence polarization of the compounds. Whether or not oxidation of the substrate occurred would depend largely on the relative magnitude of two factors: (*1*) the quantum of energy $h\gamma$ transferred from the enzyme to the substrate and (*2*) ΣEp which is determined by the resultant of the induced effect of substituent groups on the molecular moment of the compound. The rate of reaction would be determined by ΣEp for it of the two factors was the one deliberately varied by the use of homologous substrates.

It should be kept in mind that E is really the energy which must be supplied to the reacting substances to make them capable of chemical transformation into their products. The greater the magnitude of E the slower the reaction velocity where the other factors in the equation remain constant. Williams

[1] This is consistent with the views of Wagreich and Nelson (10) based on their findings that phenolase is capable of activating the oxidation of *p*-cresol only after the enzyme has been activated by an *o*-dihydroxy phenolic compound.

and Hinshelwood, and Nathan and Watson (11) have shown that polar groups substituted into a reactant produce effects upon reaction velocity proportional to the magnitude of the resulting polarization of the molecule. It follows that as the negative polarity induced in these compounds by the aromatic hydroxyl group decreases, the greater will be the value ΣEp or E for the reaction, and the slower will become the rate of reaction.

In terms of these general considerations we shall attempt to interpret our results. First, consider what constitutes enzyme specificity of the phenolase for *p*-phenolic as contrasted with *m* and *o* hydroxy phenyl homologs. As the phenolic hydroxyl group approaches the position of the side chain from the *p* through the *m* and the *o* position, in our series of compounds, it approaches the weakly positive pole represented by the side chain which has in it the positively polar amino group. From the structural formulas illustrating this point it may be seen that:



1. With the OH group in the *p* position both it and the *meta* directing side chain would facilitate the entrance of a second OH group at C-3 or C-5. The angle of separation of the negatively polar OH group on C-4 from the positively polar side chain is greatest, hence the polarization of the ring is maximal.[2]

2. When the OH group is in the *m* position its orienting effect is opposed by that of the side chain. Also its induced effect on the polarization of the molecule is decreased since it has been brought more into the field of influence of the positively polar side chain.

3. Where the OH group exists in the *o* position its orienting effect is again similar to that of the side chain. However, in this position the positive and negative poles of the ring are in such close proximity that their additive effect is to decrease the polarization of the ring to the lowest point for any of the three compounds.

These three points are consistent and permit the conclusion that the polariza-

[2] Actually, in the vector addition of individual moments of polar groups oriented at an angle to each other it is necessary to calculate the resultant moment of the molecule from the cosine law. The group moments are vectorially added by use of the equation

$$\mu = \sqrt{\mu_1^2 + \mu_2^2 + 2\,\mu_1\mu_2 \cos\theta}$$

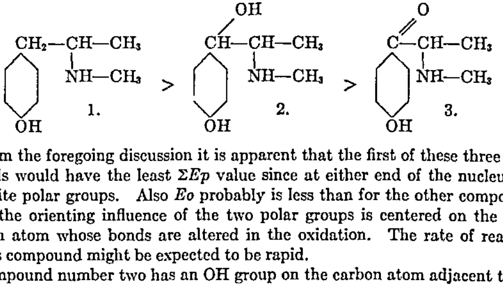
where μ is the molecular moment, μ_1 and μ_2 are the moments of the groups at C-1 and C-4, C-3 or C-2 respectively, and θ is the angle of separation of the dipoles having these moments. θ becomes 60° for the *o*, 120° for the *m*, and 180° for the *p* configurations. Substituting actual or arbitrary values for μ_1, the + polar group at C-1, and for μ_2, the − polar group, where $\mu_2 > \mu_1$ the molecular moment of the ring decreases with the angle of separation of the polar groups. See Gilman (12) for a discussion of this point.

tion of the ring is greatest when the OH group is in the *p* position, hence in this compound ΣEp would have the least energy value. It seems likely that Eo may be decreased in the *p*-phenolic compounds because of the coincidence of orientation effect of the directing groups, decreasing the energy of activation of the bond which is being acted on in the oxidation. Thus, if the additive effect of the quantum of energy transmitted from the enzyme to the substrate should be sufficient to bring about the oxidation of any of these three compounds, the *p*-hydroxyphenylethylamine would be the one most likely oxidized since the energy of activation for its reaction is least.

Since only the *p*-phenolic homologs were oxidized we have no data on the relative reactivities of *m* and *o* hydroxy compounds. Data supporting the order of reactivity as we have presented it are to be found in the work of Soper and Smith (13). They found the chlorination velocities of *o*, *m*, and *p*-cresols under identical conditions to be as follows: *o*-cresol 1.25×10^5, *m*-cresol 4.4×10^5, *p*-cresol 9.0×10^5; or *o*-cresol reacted the slowest, *p*-cresol the most rapidly.

The enzyme specificity of phenolase for the activation of oxidation of *p*-hydroxyphenyl sympathomimetic amines is seen, then, to be capable of interpretation in terms having recognized experimental basis in the physical aspects of organic chemistry.

Our second problem is to interpret the variations in rate of oxidation of *p*-phenolic sympathomimetic amines with changes in their structure. It will be recalled that the rate of oxidation of these compounds decreased from left to right according to the following formulas:



From the foregoing discussion it is apparent that the first of these three compounds would have the least ΣEp value since at either end of the nucleus are opposite polar groups. Also Eo probably is less than for the other compounds since the orienting influence of the two polar groups is centered on the same carbon atom whose bonds are altered in the oxidation. The rate of reaction of this compound might be expected to be rapid.

Compound number two has an OH group on the carbon atom adjacent to the ring. The sign of this group and its approximate dipole moment are -1.83×10^{-18}. The effect of this is to reverse the apparent polarity of the side chain, thus decrease the potential difference between the two polar groups on the ring, and so decrease polarization of the nucleus. Hence the rate of oxidation is slower.

The third compound presents an exaggeration of the same influences present in the second compound. Here a ketone group whose sign and approximate dipole moment are -2.79×10^{-18} is substituted on the carbon atom adjacent

to the ring. The presence of the ketone in this position so alters the polarity of the side chain that different fairly strongly negative polar groups are present at the two extremes of the ring. Individually each would tend to orient the introduction of a group entering the ring ortho or para to itself. But there is very likely little or no orienting influence since the vector addition of these dipoles would give a molecular moment approximating zero. Thus the value of E for the oxidation of this compound is so great that the energy $h\gamma$ transferred to it from the enzyme is apparently insufficient to bring about a rearrangement of the molecule—no oxygen uptake occurs.

The slow rate of reaction of the tertiary amino compound, hordenine, is also readily explained. Because a primary or secondary amino group is unsymmetrical it exhibits a dipole moment and so the side chain to which it is attached takes on its positive sign and a part of its moment. But in hordenine the tertiary amine induces in the side chain no measurable dipole moment, for the trivalent substitution of similar alkyl radicals makes the group practically symmetrical. Hence the side chain carries very little charge. The effect of this is to decrease the polarization on the ring below that for the corresponding primary or secondary amino compound. On theoretical basis alone the rate of oxidation of hordenine should be between compounds 1 and 2 which illustrated the influence of substitution in the side chain on rate of reaction.

The explanation for the reactions of the *o*-dihydroxyphenyl sympathomimetic amines falls naturally into this general interpretation. It is well known that compounds having this catechol nucleus are very unstable in water at pH 7.0 even in darkness and in the cold room, especially if there is a trace of iron or copper in the solution. The two hydroxyl groups on adjacent carbon atoms so greatly increase the polarization, decrease E, that only a minimal quantum of energy from any of many sources must be transmitted to such a reactant to activate its oxidation. The additive polar effect of these two adjacent OH groups is so great that the introduction of an OH or even ketone group on the alpha carbon atom of the side chain hardly serves to decrease the polarization of the ring enough to alter the rate of oxidation of these compounds significantly.

SUMMARY

In terms of the physical aspects of the enzymatic oxidative reactions which took place we have attempted to interpret the following observations.

1. Phenolase was capable of initiating the oxidation of only *p*-phenolic sympathomimetic amines as contrasted with *m* and *o* homologs which were not oxidized in its presence.

2. *p*-phenolic sympathomimetic amines having (a) a tertiary amino group or (b) an hydroxyl group on the carbon atom adjacent to the ring were less rapidly oxidized than the *p*-hydroxyphenylethylamine homologs, in the order given.

3. Where a ketone group existed on a carbon atom of the side chain alpha to the ring the *p*-phenolic nucleus did not undergo oxidation.

4. The *o*-dihydroxy phenolic compounds were oxidized as a unimolecular reaction and at a rate dependent on the concentration of substrate in solution at a given time.

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THE EFFECTS OF ATROPINE, PROSTIGMIN, ADRENALINE AND CALCIUM ON THE MOVEMENTS OF THE FASTING HUMAN STOMACH

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A considerable amount of work has been published on the effects of various drugs on gastric motility but the findings on the human stomach, of great importance therapeutically, are varied. In the course of an investigation on gastric motility in the human subject, a modification of Carlson's balloon method (Anderson 1942) was found to yield apparently consistent and reliable results and the opportunity was taken to investigate the actions of various drugs on the movements of the fasting stomach in man. The drugs dissolved in saline were given subcutaneously, intramuscularly or intravenously during different phases of gastric activity.

The effect of any stimulus on the motility of the stomach varies according to conditions existing at the time. If the stomach is showing active contractions the effect may be (a) cessation (b) increase in frequency or amplitude or (c) no alteration. Since the phase of contractions normally varies in duration the result of a single observation is worthless in determining the action of the stimulus. Accordingly the following principles have been adopted in drawing conclusions from the results of the various investigations. If a drug given early in the contraction phase is followed on every occasion by a cessation of contractions, it is concluded that the pharmacological effect is sedative. Since the frequency and amplitude of individual contractions remain practically unaltered during any one phase, a drug which uniformly causes an increase in frequency and amplitude may be said to have a stimulating action. Similarly if a drug given in a period of tonus change is always followed by the appearance of contractions an excitatory effect may be assumed but since tonus change often passes into a stage of relative quiescence no conclusion can be drawn when this occurs. Finally the consistent onset of contractions following the administration of a drug during the period of relative quiescence is assumed to indicate a stimulating effect. On many occasions injections of normal saline were given by various routes without having any apparent effect on gastric motility.

1. Atropine. It is generally accepted that atropine prevents the effect of the acetylcholine liberated at the nerve-endings of post-ganglionic fibers of the parasympathetic system. This should produce a reduction of gastric motility and an acceleration of the heart-rate. The results which have been recorded on the human subject are not, however, entirely consistent. In man, Lasch (1922) stated that increased peristalsis was generally inhibited but that normal movements still persisted. Tetelbaum (1926) found that 0.3–0.5 mgm. of atropine stopped contractions of the pyloric part although sometimes a larger dose was necessary. Neidhart (1933) in his first series of experiments found that 0.5 mgm. subcutaneously had not a constant effect and later (1935) stated that the same dose given intravenously produced a short period of paralysis. Danielopolu

(1930) on the other hand stated that 0.05 mgm. intravenously caused an increased tone and amplitude of gastric contractions, accompanied by a slowing of the heart-rate, while a second and larger dose of 0.25 mgm. inhibited movements.

In the present investigation the range of dosage was 0.05 to 1.0 mgm. of atropine sulphate intravenously and 0.6 to 2.0 mgm. subcutaneously.

Intravenous administration. Intravenous doses of 0.05 to 0.30 mgm. produced in eight patients without clinical or radiological evidence of gastro-intestinal disease an increase of gastric movements in the fasting stomach, and a slowing in the heart rate of 3 to 10 beats per minute. When hunger contractions were present at the time of injection these were increased in strength and frequency, while tonus rhythm was converted into active contractions. In one patient in whom the stomach was in a phase of relative quiescence at the time of injection, no change was noted in gastric motility but the pulse was slowed.

Doses of 0.4 to 1.0 mgm. given intravenously in seven normal subjects always produced an immediate cessation of the gastric movements. In one case the heart-rate was slowed by four beats per minute over a period of thirty minutes; in the remainder an increase of 18 to 27 beats per minute was noted.

Subcutaneous administration. Atropine sulphate 0.6 mgm. increased gastric motility in three normal subjects after a latent period of 10 to 20 minutes. In three patients with duodenal ulcer the effect was excitatory after an interval of 5 to 15 minutes, in two neutral and in one inhibitory. In two patients with gastric ulcer motility was unaltered. Within 15 minutes the heart-rate was in 8 cases slowed by 5 to 10 beats per minute for a period of 40 to 70 minutes: in two patients with duodenal ulcer no change was noted and in one healthy subject there was a preliminary slowing of 7 beats per minute for 30 minutes, followed by an acceleration of 5 beats over the basal rate lasting for the next 40 minutes.

1.0 mgm. did not appear to have any effect on the gastric motility or on the pulse-rate.

1.2 mgm. constantly produced in five healthy subjects after a latent period of 2 to 20 minutes a cessation of gastric contractions lasting for longer than 90 minutes. There was always a preliminary slowing of the heart rate of 2 to 4 beats occurring within 10 minutes followed by a gradual acceleration ranging from 13 to 32 beats over the basal level 30 to 45 minutes after the injection.

2 mgm. completely inhibited the movements of the stomach in one healthy subject, one patient with gastric ulcer and one patient with duodenal ulcer in whom a dose of 0.6 mgm. strengthened gastric contractions. In all three cases the effect on the pulse-rate was the same as that following a dose of 1.2 mgm. It is probable that the preliminary reduction of heart-rate following large subcutaneous doses of atropine is to be explained by the relatively slow absorption of the drug in contrast to the rapid distribution which occurs after intravenous injection.

Bastedo (1936) stated that in man even the most severe gastric spasm yields to large doses of atropine but that in the amounts usually employed little effect

is produced in the motor or secretory functions of the stomach. The minimum effective dose, according to him, is one milligram of atropine sulphate by subcutaneous injection. According to the results here recorded a dose of at least 1.2 mgm. subcutaneously or 0.4 mgm. intravenously is required to produce quiescence of the empty stomach.

Summation effect. Danielopolu (1930) stated that in gastric atony while 0.5 mgm. of atropine produced an improvement in tonus, a second injection caused less excitation and a third dose was followed by inhibition. In the present investigation the effect of repeated doses of atropine was studied in three subjects with healthy stomachs. 0.10 mgm. was given intravenously and repeated 30 minutes later.

In each case the first injection was followed by a reduction in the pulse-rate of 4 to 10 beats per minute and an increase in the amplitude and frequency of the hunger contractions. The second dose (0.1 mgm. in two cases and 0.05 mgm. in the third), however, was followed by an increase in the pulse-rate of 7 to 17 beats per minute and a complete cessation of gastric contractions. In two of these subjects 0.2 mgm. was given intravenously in one dose: this was followed by an increase in the frequency and amplitude of the contractions in both cases. A slight reduction in heart-rate of 3 beats per minute was noted in one subject while in the other it was unaltered. Since this is the usual response to a single injection of 0.2 mgm. of atropine it is clear that when divided doses are given the first sensitizes the individual to atropine with the result that a very small second dose leads to an effect generally produced by an amount much larger (0.6 mgm.) than the sum of the two doses. This finding suggests the interesting clinical implication that for anti-spasmodic effect atropine is best given in divided doses.

Mode of action. It is accepted that atropine in large doses has an anti-parasympathetic action. The results described here show that as far as gastric motility and heart-rate are concerned small amounts actually produce a parasympathomimetic effect. It is of course possible that this action, which is relatively mild, may be due to an inhibition of the sympathetic. Section of the splanchnic nerves, however, has no effect on gastric motility (Cannon 1911) and it is doubtful whether paralysis of the sympathetic would produce slowing of the heart since bilateral excision of the stellate ganglia does not appreciably alter the heart-rate. Bastedo (1932) and Clark (1940) attribute the inhibitory effect of small doses of atropine on the heart-rate to stimulation of the vagus centre. If this is correct one would have to assume that the medullary centre is stimulated by small doses of atropine and that larger amounts of the drug are required to produce the peripheral antiparasympathetic action. Furthermore, the effect on gastric motility would also have to be attributed to a central stimulating action and a peripheral inhibitory one. Sachs (1936) on the other hand believes that atropine is amphotropic but predominantly vagotropic. He states that in small doses it stimulates and in large doses it inhibits both systems but that the action on the parasympathetic is so much more powerful that one can neglect the effect on the sympathetic. It is generally stated that all the mus-

carinic effects of acetyl choline are prevented by atropine, while the nicotinic effects are not influenced. Marazzi (1939), however, has shown that atropine depresses or completely prevents the passage of an impulse through the synapse in an autonomic ganglion. These impulses both in the sympathetic and parasympathetic systems may therefore be blocked. The results obtained in the present investigation can be explained on the assumption that synapses in the sympathetic nerves are more readily blocked than the parasympathetic-effector junction. Thus the effect of a small dose of atropine would be to paralyse the sympathetic with consequent reduction of the heart-rate and increase of gastric motility. An alternative explanation is that small doses of atropine stimulate the parasympathetic while large doses paralyse this system.

It is usually held that atropine acts by preventing acetylcholine from entering the effector cells and stimulating the receptor substance. In this view, however, it is difficult to explain the summation effect.

The effect of a small dose of atropine is two-fold: (*1*) a parasympathomimetic action and (*2*) a sensitising effect whereby an antiparasympathetic action is obtained from a second small dose.

2. Prostigmin. Aeschlimann and Reinert (1931) showed that prostigmin, a synthetic analogue of physostigmine, has pharmacological properties similar to those of the latter drug. Following its administration choline esterase is inhibited with results generally corresponding to those following stimulation of the parasympathetic system. Fraser (1938) gives a comprehensive account of the action and uses of prostigmin.

In the present investigation the effect of the drug was studied on gastric motility and heart-rate in man. 0.5 mgm. given intravenously on eight occasions in three subjects produced a marked increase in the amplitude and frequency of gastric contractions commencing within 4 to 12 minutes. In every case slowing of the pulse commenced within 5 minutes and reached its maximum of 6 to 21 beats per minute in 10 to 25 minutes after the injection. Veach, Lauer and James (1938) as a result of their investigations in man stated that (*i*) prostigmin is usually inhibitory to gastric activity, (*ii*) the inhibitory effect of atropine on the human stomach is changed into excitation by prostigmin and (*iii*) atropine changes the inhibitor effect of prostigmin to a marked motor action. The opportunity was taken to repeat this work. At first two subjects were given 0.5 mgm. prostigmin intravenously and after 20 minutes 0.4 mgm. of atropine sulphate by the same route. The interval of 20 minutes was chosen because in our experience a latent period up to least 12 minutes may occur before the motor effect of prostigmin is demonstrated. In both instances prostigmin had its usual excitor action on gastric motility while atropine exerted a sedative effect. The pulse-rate in each subject was slowed both after prostigmin and after atropine. It has been shown that 0.4 mgm. of atropine given intravenously is ordinarily followed by a quickening of the heart-rate. It is possible that the strong parasympathomimetic effect of the prostigmin has altered the action of the atropine on the heart-rate to one corresponding to vagal stimulation. The strong sedative action of atropine on the stomach is probably due to the great

motor activity produced by the prostigmin since Meyer and Gottlieb (1926) found that the higher the preceding state of gastric tonus the more pronounced was the action of atropine.

In order to investigate the effect of previous atropinisation on the action of prostigmin, 1.0 mgm. of atropine sulphate was given intravenously and fifteen minutes later 0.5 mg. prostigmin by the same route. Following the atropine there was an immediate cessation of gastric movements and an acceleration of the heart-rate. The subsequent injection of prostigmin had no apparent influence on gastric motility but did cause a slowing of the pulse-rate. It is obvious that while atropine blocked the parasympathomimetic effect on the gastric nerves it did not do so in the cardiac nerve-endings. Certainly it did not lead to an excitor action after prostigmin. This, however, might be explained by the large dose of atropine which was used. Accordingly the same experiment was repeated using 0.6 mgm. atropine sulphate, followed 20 minutes later by 0.5 mgm. prostigmin and 30 minutes afterwards by a second dose of 0.6 mgm. of atropine. The first dose of atropine given during a period of gastric motility produced the usual sedation, the prostigmin was followed by active contractions while the second injection of atropine had a sedative effect. The pulse-rate was accelerated by the first dose of atropine, slowed by the prostigmin and again quickened by the second injection of atropine. In short neither the prostigmin nor the atropine had their effects reversed by previous injection of the other drug. It is apparent that these results are not in harmony with conclusions drawn by Veach *et alii*. The five patients used by these workers had all been suffering from some abdominal complaint while in the present investigation the three subjects were all enjoying good health and had not previously had any abdominal disorder requiring medical or surgical treatment. It may well be that the stomach of a patient with intra-abdominal disease reacts to stimuli and drugs in a different way from that of the healthy subject. Analysis of their published tracings indicate, however, that the interval allowed to elapse between the injection of prostigmin and the subsequent administration of atropine was probably too short to permit the occurrence of the prostigmin excitor effect prior to the injection of atropine. It will be remembered that in our series the increase in gastric motility after prostigmin took 4 to 12 minutes to appear while in the experiments of Veach *et alii* the prostigmin-atropine interval varied from 3 to 7 minutes. It is therefore reasonable to deduce that the so-called motor effect of atropine was really the response to prostigmin which did not become manifest till after the administration of atropine.

In order to test the validity of this hypothesis prostigmin 0.5 mgm. was given intravenously to a healthy subject and followed ten minutes later by an intravenous injection of atropine sulphate 0.65 mgm. No effect was noticed until two minutes after the administration of atropine, when there was a marked increase of gastric activity and a slowing of the heart-rate from 62 to 54 beats per minute. The ordinary effect of an intravenous dose of atropine exceeding 0.4 mg. is to produce an immediate acceleration of the heart, so that both increase of gastric movements and the decrease in heart-rate are reasonably

to be attributed to the action of the prostigmin. It would appear that in the human subject an intravenous dose of 0.65 mgm. of atropine sulphate does not prevent the parasympathomimetic action of prostigmin (0.5 mgm.)

When 0.1 mgm. of atropine was used either before or after the prostigmin, a motor effect was produced which appeared to be due to the combined action of prostigmin and atropine. This of course is what could have been expected since it has been shown that atropine in amounts less than 0.4 mgm. has an excitor effect on gastric motility. One subject received 0.1 mgm. of atropine followed 35 minutes later by 0.5 mgm. of prostigmin and 25 minutes later by 0.4 mgm. of atropine all by the intravenous route. The first injection of atropine led to an increase in gastric activity with a fall in pulse-rate, the prostigmin produced a still greater increase in the amplitude of the gastric contractions while the second dose of atropine was followed by an immediate cessation of gastric contractions.

From the results it seems reasonable to conclude (*i*) that atropine does not alter the stimulating action of prostigmin on gastric activity and (*ii*) that the atropine effect is not reversed by previous administration of prostigmin.

3. Adrenaline. Bennett (1923) stated that adrenaline, given hypodermically, had no significant action on gastric motility. Tezner and Turolt (1921) found that it had an inhibitory effect on the surviving human stomach kept in physiological saline. Carlson (1916) and Dickson and Wilson (1925) maintained that adrenaline reduced the gastric movements. Danielopolu (1930), on the other hand, found that adrenaline given intravenously in very small doses increased gastric motility and in large doses diminished the contractions of the stomach.

In the present investigation doses of 0.0025 to 0.025 mgm. were given intravenously and 0.6 to 1.0 mgm. subcutaneously. The subjects of the investigation were six healthy adults without evidence or history of gastric disorder.

An intravenous injection of 0.0025 mgm. had no effect on gastric motility in four subjects but in two caused a slight increase in the frequency and amplitude of the gastric contractions. In two of the former group there was a slight reduction of the heart-rate amount to three and four beats per minutes respectively, while in the remainder no effect on the heart was detected. With a dose of 0.005 mgm. a slight sedative effect on gastric motility was noticed in one healthy subject but no change in the pulse-rate was observed. With doses of 0.01 and 0.025 mgm. the sedative effect was more marked, amounting on several occasions to complete cessation of movements while the pulse-rate was sometimes quickened and sometimes unchanged. These results would seem to furnish slight but not very convincing support to the view of Danielopolu (1930) that adrenaline in very small doses tends to stimulate the parasympathetic nerves and in larger doses the sympathetic. When larger amounts (0.6 to 1.0 mgm.) of adrenaline were given subcutaneously to four subjects the results in every instance were complete cessation of gastric movements lasting for 15 minutes to more than an hour and slight acceleration of the pulse-rate (3 to 4 beats per minute).

4. Calcium. The effect of calcium salts on gastric motility has received the attention of workers with variable results. Carlson (1916) stated that calcium chloride produces a temporary depression whereas Dickson and Wilson (1928) found increased peristalsis. Danielopolu (1930) observed that while smaller doses of calcium chloride caused either an inhibition or a transient increase of gastric motility, larger doses always produced an inhibition. Neidhart (1935) found that the intravenous injection of 5 to 10 cc. of a 1% solution of calcium chloride was followed by a cessation of gastric movement lasting from 10 to 30 minutes.

In the present investigation calcium gluconate was given intravenously in 10 cc. doses of a 10% solution to seven patients with healthy alimentary tracts. In four the injection of calcium was followed within 6 minutes by a complete cessation of gastric contractions which lasted for at least 10 minutes. In the remaining three subjects, both the frequency and the amplitude of contractions were reduced. In all, the pulse-rate was diminished by four to six beats per minutes.

Calcium gluconate did not influence the usual sedative effect of an intravenous injection of 0.6 mgm. atropine given 40 minutes later. When, however, the dose of atropine was reduced to 0.1 mgm. its usual excitor effect was held in check presumably by the calcium for a period of about ten minutes when it became apparent. In every instance, however, the small dose of atropine caused a further slowing of the pulse-rate within 5 minutes. On the strong gastric contractions produced by prostigmin (0.5 mgm.) calcium gluconate administered ten minutes later had no effect. When given fifteen minutes before prostigmin the calcium salt was unable to prevent or reduce the motor activity caused by the parasympathomimetic drug. On the gastric motility calcium gluconate in the amount given has the same effect as the larger doses of atropine whereas its action on the heart-rate is similar to that produced by smaller doses of atropine. Compared with prostigmin it was found that both drugs had the same effect on the heart-rate but differed in their actions on gastric motility.

Rogen (1940) suggests that the effect of calcium on the heart is due to its stimulating action on the vagus while Sachs (1936) states that calcium stimulates the sympathetic system. One explanation of the results recorded here is that calcium stimulates the parasympathetic nerve supply of the heart and the sympathetic fibers to the stomach. It is possible of course that the gastric effect is due to paralysis of the parasympathetic but this seems less likely in view of the fact that the inhibitor effect of calcium is weak compared with that of atropine.

SUMMARY

The action of various drugs on the motility of the fasting stomach in man is recorded.

1. Atropine in small doses has a parasympathomimetic action causing an increase in the frequency and amplitude of the hunger contractions and a slowing of the pulse-rate. This dosage has also a sensitising effect whereby an anti-

parasympathetic action is obtained from a second small dose. In large doses atropine inhibits the movements of the stomach and quickens the heart-rate.

2. Prostigmin has been shown to have excitor effect on gastric motility. Premedication with atropine does not change this action. If given in large doses, however, the atropine may prevent the stimulating effect of the prostigmin. The effects of atropine are not altered by previous administration of prostigmin.

3. Adrenaline in very small doses occasionally appears to increase gastric motility. Large doses constantly have a sedative action.

4. Calcium has a sedative action on the fasting contractions of the stomach and slows the pulse-rate.

We desire to express our indebtedness to the late Dr. William Martin, late Medical Superintendent, Stobhill Hospital, for the facilities afforded to us and to the Medical Research Council for a grant towards expenses.

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RELATION OF THE EXTRINSIC NERVES OF THE INTESTINE THE INHIBITORY ACTION OF ATROPINE AND SCOPOLAMIN ON INTESTINAL MOTILITY[1]

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The following experiments were undertaken to determine the mechanis the inhibition of the motility of the intestine *in situ* by atropine. The re show that (*1*) the inhibitory action of atropine on the intestine is exerted i pendently of the extrinsic nerves, (*2*) the intestine is sensitized to atropin certain denervations, and (*3*) the inhibition is not produced because of libera of adrenalin and sympathin. Facts are presented in support of the interpr tion that atropine inhibits the extrinsically denervated intestine by preven the action of acetylcholine which is being produced constantly in the intes independently of impulses from extrinsic nerves. The results also indicate t atropine inhibits the innervated intestine by the same mechanism.

METHODS. The motility of jejunal segments in the form of Thiry fistulae was reco by the balloon-mercury-manometer method in unanesthetized dogs. This method sti lates rhythmic contractions which continue at four or five second intervals for hours. length of the balloons used was 5 cm. and the volume of balloon and contents was appr mately 6 to 8 cc. The kymographic records show pressure changes which are recorded f a mercury manometer. The method records contractions which are virtually isome since a large variation in pressure is recorded with only a small reduction in volume. U these conditions all loops, regardless of the presence or absence of extrinsic innervat show strong rhythmic contractions. In all cases the loops maintain pressure against balloons even in the "diastolic" phase. The minimal pressure ranges from a few to 1 20 mm. Hg above the intra-abdominal pressure. The loops lacking sympathetic inne tion tend to show a higher "tonus," possibly because of elimination of continual inhibit influences and certainly because of elimination of the intestino-intestinal inhibitory re No clear differences are observed between records taken under basal conditions from nor or from vagotomized animals.

Two fistulae were made from adjacent segments of the jejunum in each animal. Sin taneous records were taken from each of the two segments by the use of identical record systems. The relation of the action of atropine and scopolamine to innervation was termined as follows: (*1*) Normal innervation; (*2*) All extrinsic nerves cut in the mesente (*3*) Vagal innervation destroyed at the level of the lower esophagus; (*4*) sympathetic c nections cut between the pre-aortic ganglia and the central nervous system by splanc cotomy and removal of the lumbar sympathetic chains; (*5*) Combination of (*3*) and and (*6*) Combination of (*2*), (*3*) and (*4*). In most cases the preparation was such that two segments in the same animal differed from each other with regard to only one se nerves so that the relation of this set to the sensitivity of the intestine to the inhibitory tion of atropine could be determined.

The procedure for performing an experiment was as follows. The dog was allowed lie unrestrained on a table while pressure changes were recorded from each of the intesti

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[2] Research Assistant on a grant from the General Research Council of the Oregon St System of Higher Education.

segments. No drug other than that being tested was administered. After a period long enough for the heart rate to become constant and to allow for adjustment of the intestinal segments to the introduction of the balloons, the heart rate and intestinal motility were recorded for ten or fifteen minutes. Then 0.1 mgm. per kilogram of atropine sulfate or scopolamine hydrobromide dissolved in one or two cc of distilled water was injected subcutaneously, and the record was continued until the full effect of the drug on intestinal motility and heart rate was demonstrated. In some cases the record was continued into the recovery period. Heart rate was determined from time to time by counting the apex beat.

The present study includes analysis of 30 records from 6 dogs However, the inhibitory action of atropine or scopolamine upon extrinsically denervated intestinal segments has been observed in 7 dogs in former studies (1, 2) and in numerous demonstrations in the teaching laboratory.

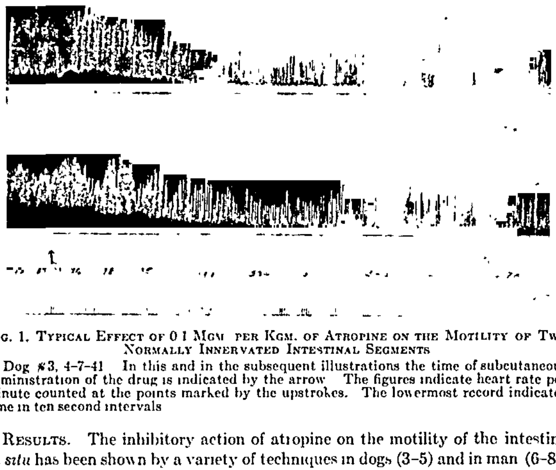

FIG. 1. TYPICAL EFFECT OF 0 1 MGM PER KGM. OF ATROPINE ON THE MOTILITY OF TWO NORMALLY INNERVATED INTESTINAL SEGMENTS

Dog #3, 4-7-41 In this and in the subsequent illustrations the time of subcutaneous administration of the drug is indicated by the arrow The figures indicate heart rate per minute counted at the points marked by the upstrokes. The lowermost record indicates time in ten second intervals

RESULTS. The inhibitory action of atropine on the motility of the intestine *in situ* has been shown by a variety of techniques in dogs (3–5) and in man (6–8). In the present study the subcutaneous injection of 0 1 mgm. per kilogram of atropine or of scopolamine resulted, after a latent period of a few minutes, in marked or complete inhibition of rhythmic contractions of the intestinal segments and in decreased pressure within the lumen. Typical records of the effects of the drugs are shown in figures 1 and 2. These records also serve to illustrate the similarity of the effects on the motility of two separate intestinal segments in the same animal when the segments have identical innervation. A third fact shown in figures 1 and 2, which are obtained from the same dog, is the similar degree of intestinal inhibition that is produced by equivalent doses of the two

drugs. The effects of atropine and scopolamine were found to be similar throughout this study; therefore, for the sake of brevity, only atropine will be mentioned in the discussion.

I. *Effect of complete extrinsic denervation of the intestine on the action of atropine on intestinal motility.* Evidence that atropine exerts its action on intestinal motility independently of extrinsic nerves is derived from the fact that the intestine-inhibiting action of atropine is undiminished by complete extrinsic denervation. In fact the denervated intestine is inhibited more than the innervated segment. The type of record obtained is illustrated in figure 3. The hypersensitivity to atropine in the denervated segment is manifested in an

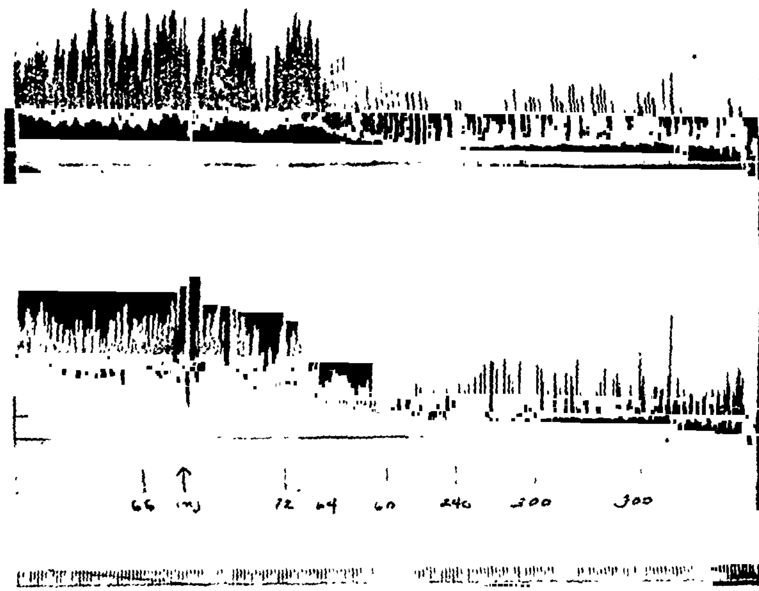

FIG. 2. TYPICAL EFFECT OF 0.1 MGM. PER KGM. OF SCOPOLAMINE ON THE MOTILITY OF TWO NORMALLY INNERVATED INTESTINAL SEGMENTS. DOG #3, 4-18-41

earlier onset of the inhibition, greater degree of inhibition during the time of maximal effect, and slower recovery from the inhibition. The inhibition of the denervated intestine must be caused by the action of a blood-borne substance upon the neural or muscular elements in the intestinal segment. The inhibitory substance could be atropine itself or a substance produced somewhere in the animal by the action of atropine. It has been reported that administration of relatively large doses of atropine will cause liberation of adrenalin (9) and sympathin (10). Records such as that illustrated in figure 3 could possibly be obtained if the sole action of atropine were to cause the liberation of sympathomimetic substances, since denervation of the intestine sensitizes it to the inhibitory action of adrenalin (11, 12). Comparisons of the sensitivity of the denervated

heart and of the denervated intestine to adrenalin in unanesthetized dogs have shown that the amount of adrenalin required to produce prolonged complete intestinal inhibition under these conditions is sufficient to produce, simultaneously, marked cardiac acceleration (13, 14). Although it requires only a very small amount of adrenalin to produce a period of inhibition of the denervated intestine, a relatively large amount is required to keep the intestine inhibited for a prolonged period. Sympathomimetic substances are not liberated in these experiments in quantities sufficient to have effects after circulatory removal from the site of production. This is indicated by the fact that the dose of atropine

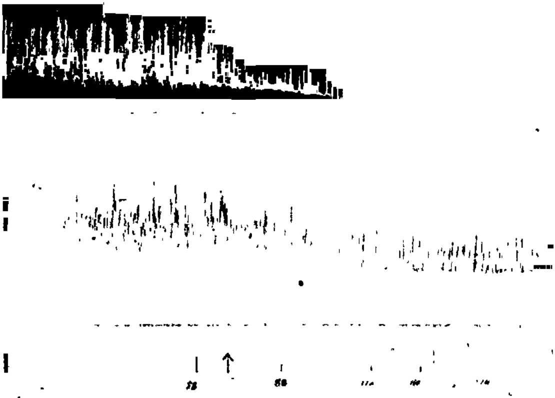

FIG. 3 COMPARISON OF THE EFFECT OF 0 1 MGM. PER KGM. OF ATROPINE ON THE MOTILITY OF AN INNERVATED SEGMENT (LOWER RECORD) AND A DENERVATED SEGMENT (UPPER RECORD) OF THE INTESTINE
Time in 5 second and one minute intervals. Dog #4, 1-6-38

required to produce complete inhibition of the denervated intestine has been found to have no effect on the rate of the denervated heart. Furthermore, the animals having their splanchnic nerves cut and lumbar chains removed have their adrenal glands denervated and the larger part of their adrenergic nerves destroyed, yet the denervated intestine in these animals is inhibited by atropine to as great a degree as in any other preparation. The intestine-inhibiting action of atropine is also seen in animals with the adrenal medullae removed by cautery. In favor of a local action of the atropine itself are reports of hypomotility of intestinal segments removed from atropinized animals (15) and of inhibition of isolated intestinal segments by atropine in high dilutions (16).

If the inhibition of the extrinsically denervated intestine is to be attributed to a local action of atropine it is still possible that the atropine is causing the inhibition by its ability to interfere with the action of acetylcholine. The most likely interpretation of the mechanism by means of which atropine inhibits the denervated intestine is that the level of motility observed before administration of atropine is sustained by the stimulant effect of acetylcholine produced in the intestine independently of extrinsic nervous effects and that atropine blocks the effect of this acetylcholine. There is evidence that the denervated or innervated intestine *in situ* (17) and the isolated intestine contracting in Locke's solution (18) or minced (19), produces acetylcholine in large quantities relative to other tissues. The small intestine exceeds much of the purely nervous tissue with regard to content of "bound" acetylcholine (17) and acetylcholine-esterase (20). Studies that have demonstrated production of acetylcholine in the intestine during stimulation of the peripheral end of the cut vagus also give evidence for a basal production of acetylcholine before the stimulation (21). It is evident that the intestine contains the biochemical mechanisms for production and destruction of acetylcholine and that these mechanisms operate at a basal level in the absence of stimulation by extrinsic nerves. Although the amount of atropine used in this study is not sufficient to block the effects of vagal stimulation on intestinal motility, it is sufficient to decrease or prevent the intestinal effects of injected choline derivatives. This interpretation of the mechanism of action of atropine is based on the assumption that the intrinsic acetylcholine metabolism of the intestine has a physiological rôle in the regulation and perhaps in the initiation of the rhythmic contractions of the intestine. Experiments are in progress to test the implications of this assumption. The mechanism by means of which atropine inhibits the innervated intestine under basal conditions is most likely the same as that for inhibition of the denervated intestine, since there is minimal activity of cholinergic nerves under these conditions, and the amount of atropine used is not sufficient to block them.

II. *Effect of vagotomy and of sympathectomy on the sensitivity of intestinal smooth muscle to the inhibitory action of atropine.* Experiments were designed to determine whether it was the sympathetic or the vagal denervation of the intestine that was responsible for the increased sensitivity to atropine observed in the completely denervated intestine. The rôle of the vagus was determined by comparing the effects of the standard dose of atropine in a given animal before vagotomy with the effects of the same dose a week or two after vagotomy. This type of comparison is not as satisfactory as the comparison of simultaneous records from two loops differing from each other with regard to only one set of nerves. The results definitely indicated that the inhibitory action of atropine on intestinal motility is undiminished by vagotomy, and it is possible that a mild sensitization to atropine actually occurs following vagotomy. This result may be observed by comparing the maximal effects of atropine in the upper record of figure 1 in dog #3 (taken before vagotomy) with the upper record of figure 4B (taken after vagotomy).

Sympathetic denervation increases the sensitivity of the intestine to the inhibitory action of atropine. This is demonstrated by cutting the mesenteric

nerves to one of the intestinal segments in an animal whose vagi have already been cut. The mesenterically denervated loop shows greater inhibition in re-

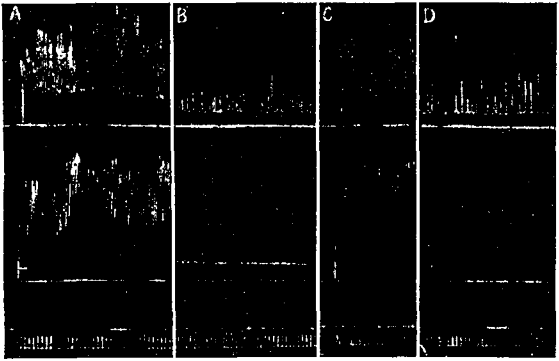

Fig. 4. Effect of Atropine and Scopolamine on the Motility of a Completely Denervated Intestinal Segment (Lower Record) Compared with Effect on a Segment Having Only Its Vagal Innervation Destroyed (Upper Record)

Dog #3. A Before injection of atropine B Maximal inhibition obtained following injection of atropine, 0.1 mgm. per kgm C. Normal motility several hours later. D. Maximal inhibition obtained after injection of scopolamine, 0 1 mgm per kgm

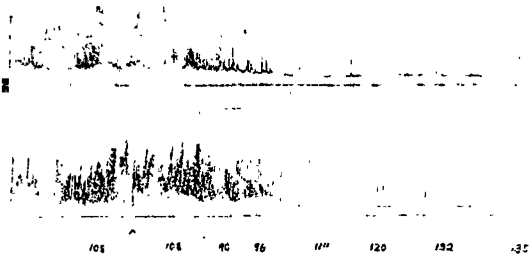

Fig. 5. Effect of Scopolamine on the Motility of a Completely Denervated Intestinal Segment (Upper Record) Compared with Effects on a Segment (Lower Record) Retaining Its Connections with the Pre-Aortic Ganglia Which Have Been Decentralized by Vagotomy, Splanchnicotomy, and Lumbar Ganglionectomy

sponse to atropine than that simultaneously recorded from the loop having only its sympathetic innervation. This result is illustrated in figure 4. The sensiti-

zation may be another manifestation of the non-specificity of the alteration in sensitivity of effectors produced by destruction of their efferent innervation, or it may be on some other basis. It is probably not related to differences in tonus of the two loops prior to the administration of the atropine.

Destruction of the nervous connections between the intestine and the decentralized pre-aortic ganglia results in an increased sensitivity of the intestine to the inhibitory action of atropine. This was shown by sectioning the mesenteric nerves to one of two intestinal segments in an animal having had its vagi and splanchnic nerves cut and the lumbar sympathetic chains removed. Simultaneous records of the effects of atropine on the two loops are shown in figure 5.

III. *Cardiac effects during the period of intestinal inhibition produced by atropine.* The amount of atropine required to inhibit intestinal motility in the unanesthetized dog is less than the minimal needed for production of cardiac acceleration. With less stimulant methods of recording intestinal motility inhibitory effects may possibly be obtained with considerably smaller doses. In the frequent cases in which a mild cardio-inhibitory action of atropine is observed prior to the onset of the acceleration, the intestinal segments may become inhibited even before the beginning of the cardiac inhibition. Heart rate is indicated at various points in figures 1, 2, 3 and 5. Henderson (22) found that the dose of atropine required to have any depressant effect on the excitatory action of vagal stimulation on intestinal motility was many times the dosage required either to decrease the cardio-inhibitory effects of vagal stimulation or to inhibit intestinal motility. These results indicate that atropine inhibits intestinal motility in doses below the amount required to depress the intestinal vagus.

SUMMARY AND CONCLUSIONS

The mechanism of the inhibitory action of atropine on intestinal motility has been investigated by the use of intestinal segments in the form of Thiry fistulae in unanesthetized dogs having had various operations on the extrinsic nerves of the intestine. Intestinal motility was recorded by the balloon-mercury-manometer method.

Atropine exerts an inhibitory action on the tonus and rhythmic motility of the intestine independently of the extrinsic nerves. Vagotomy does not reduce the intestine-inhibiting action of atropine. Sympathectomy results in an increased sensitivity of the intestine to the inhibitory action of atropine. The mechanism of the sensitization has not been determined.

The inhibition of the denervated intestine following administration of small doses of atropine is not caused by adrenalin or sympathin. The inhibition is probably accomplished through a local action of atropine on the neural or muscular elements of the intestinal wall.

The most likely interpretation of the mechanism of action of atropine is that it renders the intestinal smooth muscle unresponsive to acetylcholine which is being produced at a basal level in the intestine independently of extrinsic nerves. This interpretation implies that the acetylcholine thus produced has a physiological rôle in the maintenance of intestinal motility.

The actions of scopolamine on intestinal motility following the various denervations were indistinguishable from those of atropine.

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EFFECT OF COCAINE ON THE EXCRETION OF PHENOL

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At the present time there are two theories to explain the inactivation of epinephrine in the body. The first is that the body inactivates epinephrine by oxidation of the side chain. The second theory is the one suggested by Richter (1, 2) according to which the body inactivates epinephrine by esterification of the phenol ring. A contribution to the latter theory was made by the observation (3) that cocaine, one of the known potentiators of the effect of epinephrine *in vivo*, inhibits the phenol sulfur esterase *in vitro*. Phenol sulfur esterase is the enzyme which is assumed to be involved in the esterification of the phenol ring in the body. The purpose of this paper is to determine whether or not cocaine inhibits this enzyme *in vivo*. One way to determine the activity of the phenol sulfur esterase *in vivo* is to measure how much introduced phenol is eliminated as conjugated sulfur ester in the urine. A decrease in this process of esterification resulting from the presence of cocaine in the body would be evidence in favor of inhibition of the enzyme by cocaine *in vivo*.

METHODS. A series of fifteen experiments was performed. Each experiment used eight cats individually caged. The cats were not fed during the whole experiment because food intake may influence the phenol metabolism. After three days of fasting two cats were injected subcutaneously with 5 mgm./kg. phenol, and two cats first with 15 mgm./kg. cocaine and ten minutes later with 5 mgm./kg. phenol. Two uninjected cats and two cats injected with 15 mgm./kg. cocaine served as controls. Each cocainized cat was reinjected every two hours with 2 mgm./kg. cocaine for a period of 24 hours. The urine of each cat was collected for 7 days in containers and once a day the content of the bladder was emptied manually. The free and conjugated phenol contents of each 24 hour sample were determined by the method of Theis and Benedict (Marenzi, 4). The color changes due to the phenol content of each sample were measured with a photoelectric colorimeter. The phenol excretion was computed in mgm. per kg. per 24 hours and expressed as per cent of the value found on the last day of fasting before injection.

RESULTS. The average concentration of phenol eliminated in the urine was 45 mgm. per 100 cc., and the 24 hour excretion 6.7 mgm. free and 4.5 mgm. conjugated phenol per kg. of body weight. The elimination of phenol decreased slightly during the first week of fasting. Injection of cocaine alone did not modify significantly the daily elimination of phenols. The greater part of the injected phenol was eliminated, partly in free and partly in conjugated form, during the first 24 hours. After injection of phenol fasting cocainized cats eliminated the same amount of conjugated phenol as uninjected animals, but eliminated slightly more free phenol. These results expressed as percentage changes are shown in Table 1.

DISCUSSION. Injected phenol is eliminated in the urine of fasting cats during

the first 24 hours partly as free and partly as conjugated phenol. On the other hand, cocainized cats eliminate some of the injected phenol in free form but they do not eliminate it in conjugated form. (The absence of a significant change of the daily elimination of conjugated phenol after an injection of cocaine alone may be due to the lack of specificity of the chemical method for phenol.) These results suggest that cocaine inhibits the esterification of phenols *in vivo* and are in agreement with the observation that cocaine inhibits phenol sulfur esterase *in vitro* (3). They also substantiate the hypothesis that cocaine potentiates the effect of epinephrine at least in part by inhibition of enzymatic processes which

TABLE 1

Changes in the Concentration of Phenol in the Urine of Fasting Cats under Various Conditions

Each figure represents the average value for 30 cats followed by the standard error of the mean

TREATMENT	PHENOL EXCRETION IN PERCENTAGE OF CONTROL (= 100%)		
	First 24 hours	Second 24 hours	Third 24 hours
Fasting†	96 ± 5.1*	91 ± 5.0	89 ± 5.4
Cocaine	96 ± 5.1	91 ± 4.1	90 ± 4.8
Phenol	136 ± 8.5	105 ± 6.2	91 ± 4.9
Cocaine-phenol	106 ± 5.2	91 ± 4.8	90 ± 4.2
	Conjugated phenol		
Fasting†	97 ± 5 1	91 ± 4.9	90 ± 5 5
Cocaine	95 ± 4 8	90 ± 5.0	90 ± 5.4
Phenol	140 ± 7.2	106 ± 5.2	93 ± 6.2
Cocaine-phenol	96 ± 4 2	92 ± 5.0	90 ± 5.9

* S.E. of mean $= \sqrt{\frac{\Sigma(\Delta)^2}{N(N-1)}}$. ($\Sigma$ = summation, Δ = deviation of each value from the mean, N = number of experiments.

† Values related to phenol excretion during previous 24 hours (third fasting day).

are involved in the inactivation of epinephrine through esterification of the phenol ring.

SUMMARY

1. The effect of cocaine on the phenol elimination of fasting cats has been investigated.

2. Cocaine alone does not significantly modify the phenol elimination.

3. Non-cocainized cats eliminate the injected phenol during the first 24 hours.

4. Cocainized cats do not show any significant increase in the elimination of phenol in the urine after injection of phenol, indicating that cocaine inhibits the enzymatic processes involved in the esterification of phenols *in vivo*.

5. This evidence of enzyme inhibition is a further indication of the possibility of the inactivation of epinephrine by esterification of the phenol ring.

drugs, suspended in 10% acacia, were administered by stomach tube. In one test, single doses were administered 2 hours after infection; in the remaining tests, the drugs were administered 2, 8 and 14 hours after infection and at 8 hour intervals thereafter for five additional doses. Mice were kept under observation for 30 days after infection. In view of the findings of Litchfield, White and Marshall (9), this period of observation seemed adequate. In order to determine definitely whether late deaths were due to infection with streptococci, cultures of heart blood were prepared from all mice that died 10 to 30 days after infection.

In order to interpret the results of the therapeutic tests, the concentrations of the sulfonamides in the blood were determined following administration of the various doses used in treatment. Heart blood samples were obtained from groups of four to six mice 2, 4 and 6 hours after administration of a given drug; these samples were analyzed for sulfonamide content according to the method of Bratton and Marshall (10). The color comparison was made with a Klett photoelectric colorimeter, making possible reasonably accurate determinations of sulfonamide concentrations as low as 0.5 to 1 mgm. %.

RESULTS. *1. Comparative activities of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide when administered in equal doses.* The criteria used for assessing the relative activities of the different sulfonamides were: (*1*) *curative action* as shown by the numbers of mice surviving in good health for 30 days after infection, and (*2*) *life-prolonging action* as shown by the numbers of animals surviving 5, 10 and 20 days after infection.

a. Comparative activity of single large doses. The effectiveness of single 20 mgm. doses of the five sulfonamides was studied against infections with strains C203 and Schw. The results of these experiments, summarized in table 1, show that in both curative and life-prolonging activities sulfapyrazine and sulfadiazine were considerably more effective than sulfathiazole, sulfapyridine or sulfanilamide. In the experiment with strain C203 there were 13 survivors on sulfadiazine treatment and 11 on sulfapyrazine as contrasted with 9, 5 and 5 survivors on sulfanilamide, sulfapyridine and sulfathiazole respectively. In the experiment with strain Schw there were 21 survivors on sulfadiazine treatment and 18 on sulfapyrazine, whereas there were only 6, 8 and 12 survivors among the groups treated with the other three drugs.

In reference to the life-prolonging action of these sulfonamides, there were comparatively small differences in the numbers of survivors 5 days after infection; however, in both experiments the differences favored sulfapyrazine and sulfadiazine. At the end of 10 and 20 days, a significant difference in the activity of the drugs was apparent. This was most striking in the experiment with strain Schw in which there were 28 10-day survivors among the sulfapyrazine-treated mice and 22 among those treated with sulfadiazine as compared with 14, 10 and 9 survivors, respectively, among the animals in the sulfathiazole, sulfapyridine and sulfanilamide groups.

It will be noted that the relative activities of sulfapyrazine and sulfadiazine were essentially identical in both experiments. The relative activities of the other three sulfonamides varied somewhat, but there was no great difference in their activities. In the experiment with strain C203, sulfanilamide was slightly more effective than sulfathiazole and sulfapyridine, whereas sulfathiazole was the most effective in the experiment with strain Schw.

b. Comparative activity of repeated doses against infections with strain C203. The activities of the five sulfonamides, administered in repeated doses of 0.1 to 5 mgm., were studied in infections with strain C203. The results of these experiments, recorded in table 2, show that sulfapyrazine and sulfadiazine were distinctly more effective than the other sulfonamides. This conclusion is best supported by reference to the experiments in which the lower dosages were used. For example, on a dosage of 0.1 mgm., all 30 of the mice treated with sulfapyrazine and all 30 treated with sulfadiazine were alive and in apparently good health five days after infection, whereas at that time there were only three survivors in each of the groups treated with the other sulfonamides. At the end

TABLE 1

Comparative effectiveness of single doses of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide against infections with beta hemolytic streptococci, strains C203 and Schw

Dosage = 20 mgm. administered 2 hours after infection

INFECTING ORGANISM	NUMBER OF ORGANISMS IN INFECTING DOSE	NUMBER OF MICE INFECTED	SULFONAMIDE* ADMINISTERED	NUMBER OF SURVIVORS Days after infection						
				1	2	3	5	10	20	30
C203	414	30	SPZ	30	29	29	26	14	11	11
		30	SD	30	30	30	29	19	13	13
		30	ST	30	30	25	11	5	5	5
		30	SP	30	30	30	20	13	6	5
		30	SA	30	29	29	23	10	9	9
		20	Control	5	0	0	0	0	0	0
Schw	564	30	SPZ	30	30	30	30	28	18	18
		30	SD	30	30	29	28	22	21	21
		30	ST	30	30	30	27	14	12	12
		30	SP	30	30	30	20	10	8	8
		29	SA	28	27	27	20	9	6	6
		19	Control	10	0	0	0	0	0	0

* SPZ = sulfapyrazine; SD = sulfadiazine; ST = sulfathiazole; SP = sulfapyridine; SA = sulfanilamide.

of 30 days there were 12 and 13 survivors in the groups treated with sulfapyrazine and sulfadiazine respectively, as contrasted with two survivors in the group treated with sulfathiazole and none in the sulfapyridine and sulfanilamide groups.

As the dosage was increased above 0.5 mgm., the difference between the effectiveness of sulfapyrazine and sulfadiazine and that of the other drugs diminished to a certain extent. This occurred because the curative activity of sulfathiazole, sulfapyridine and sulfanilamide, almost negligible on 0.1 and 0.2 mgm. doses, increased progressively as the dosage of these drugs was raised from 0.5 to 5 mgm., whereas in the case of sulfapyrazine and sulfadiazine, 0.5 and 1.0 mgm. doses were nearly as effective as 2.5 and 5.0 mgm.

TABLE 2

Comparative effectiveness of repeated doses of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide against infections with beta hemolytic streptococci, strain C203

Indicated dosage administered 2, 8 and 14 hours after infection and at 8 hour intervals thereafter for five additional doses

NUMBER OF ORGANISMS IN INFECTING DOSE	NUMBER OF MICE INFECTED	TREATMENT		NUMBER OF SURVIVORS						
		Drug*	Dosage	Days after infection						
				1	2	3	5	10	20	30
			mgm.							
390	30	SPZ	0.1	30	30	30	30	12	12	12
	30	SD	0.1	30	30	30	30	13	13	13
	30	ST	0.1	28	21	16	3	2	2	2
	30	SP	0.1	28	26	24	3	1	0	0
	30	SA	0.1	30	25	25	3	0	0	0
	30	SPZ	0.2	30	30	30	28	13	13	13
	29	SD	0.2	29	29	29	29	18	16	16
	30	ST	0.2	30	26	25	5	1	1	1
	30	SP	0.2	30	30	29	8	6	5	5
	29	SA	0.2	29	29	29	12	2	2	2
	20	Control		0	0	0	0	0	0	0
510	30	SPZ	0.5	30	30	30	28	18	18	18
	30	SD	0.5	30	30	30	30	23	21	21
	30	ST	0.5	30	30	29	12	5	5	5
	30	SP	0.5	30	29	29	13	5	0	0
	30	SA	0.5	30	30	30	20	12	12	12
	30	SPZ	1.0	30	30	29	27	20	19	19
	30	SD	1.0	30	30	30	27	19	19	19
	30	ST	1.0	30	26	26	22	14	10	10
	30	SP	1.0	30	30	30	18	11	10	10
	30	SA	1.0	30	30	30	19	11	11	11
	20	Control		0	0	0	0	0	0	0
352	30	SPZ	2.5	30	30	30	29	22	22	22
	30	SD	2.5	30	30	30	30	28	24	24
	30	ST	2.5	30	30	30	25	12	12	12
	30	SP	2.5	30	30	30	30	17	17	17
	30	SA	2.5	30	30	30	28	19	19	19
	30	SPZ	5.0	30	30	30	30	25	22	22
	30	SD	5.0	30	30	30	28	27	25	25
	30	ST	5.0	30	30	30	29	18	18	18
	30	SP	5.0	30	30	30	24	14	14	14
	30	SA	5.0	30	30	30	29	26	22	22
	20	Control		1	0	0	0	0	0	0

* SPZ = sulfapyrazine; SD = sulfadiazine; ST = sulfathiazole; SP = sulfapyridine; SA = sulfanilamide.

That the activities of these sulfonamides tended to converge is best shown by the fact that the life-prolonging action of all five drugs was approximately equal when doses of 2.5 and 5 mgm. were administered. For example, 29 of the mice receiving 2.5 mgm. doses of sulfapyrazine and 30 of those receiving sulfadiazine were alive at the end of 5 days as compared with 28, 30 and 25 survivors in the groups treated with sulfanilamide, sulfapyridine and sulfathiazole. It is pertinent to note, however, that within the limits of the doses administered, sulfapyrazine and sulfadiazine always had greater curative activity than sulfathiazole and sulfapyridine, and only when 5 mgm. doses were used did the activity of sulfanilamide equal that of the former two drugs.

The activities of sulfapyrazine and sulfadiazine were essentially equal in each experiment, although in all but one case from one to three more mice survived on sulfadiazine treatment than on sulfapyrazine. Sulfanilamide was slightly more effective than sulfapyridine and sulfathiazole. There was no significant difference in the activities of these latter two drugs.

c. Comparative activity of repeated doses against infections with strains CF1, CF3, CF4 and CF5. Preliminary experiments showed that infections with these strains are somewhat more susceptible to sulfonamide treatment than are infections with strain C203. Therefore, in order to demonstrate differences in activity, it was necessary to use much smaller doses of the drugs than had been used in our previous experiments. The doses administered were 0.1 and 0.025 mgm. The results of the comparison are given in table 3.

Although the results were not as regular as those on strain C203, sulfapyrazine and sulfadiazine were appreciably more effective than the other three sulfonamides against infections with each of the above strains. The difference between the curative activity of these two drugs and that of sulfathiazole, sulfapyridine and sulfanilamide was most striking against infections with strain CF4. Thus, of the animals infected with this strain, 16 of those treated with 0.025 mgm. doses of sulfapyrazine and 19 treated with sulfadiazine were alive at the end of 30 days, whereas there were only three survivors on sulfathiazole treatment and two each on sulfapyridine and sulfanilamide treatment. In infections with the other three organisms, the difference between the curative actions of the drugs was less marked. However, there was a distinct difference in life-prolonging activity, particularly on the low dosage. Thus in the case of strain CF3, 27 of the mice treated with sulfapyrazine and 25 of those treated with sulfadiazine survived for 5 days as compared with 17, 10 and 9 survivors, respectively, among the groups treated with sulfanilamide, sulfapyridine and sulfathiazole.

As was the case against infections with strain C203, sulfapyrazine and sulfadiazine were approximately equal in activity. There were some instances in which sulfapyrazine was slightly more effective than sulfadiazine and other instances in which sulfadiazine was the more active. No definite conclusion could be drawn as to the relative activities of sulfanilamide, sulfapyridine and sulfathiazole, since the differences in the effectiveness of these drugs were comparatively small and the relative activities varied in experiments with different strains.

TABLE 3

Comparative effectiveness of repeated doses of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine and sulfanilamide against infections with beta hemolytic streptococci, strains CF1, CF3, CF4 and CF5

Indicated dosage administered 2, 8 and 14 hours after infection and at 8 hour intervals thereafter for five additional doses

INFECTING ORGANISM	NUMBER OF ORGANISMS IN INFECTING DOSE	NUMBER OF MICE INFECTED	TREATMENT		NUMBER OF SURVIVORS						
					Days after infection						
			Drug*	Dosage	1	2	3	5	10	20	30
				mgm.							
CF1	460	30	SPZ	0.025	29	23	23	20	16	11	10
		30	SD	0.025	30	30	29	26	21	18	17
		30	ST	0.025	14	10	8	6	5	4	4
		30	SP	0.025	22	16	15	15	14	13	13
		30	SA	0.025	22	15	15	13	11	11	11
		20	Control		0	0	0	0	0	0	0
	160	30	SPZ	0.1	30	29	29	28	26	24	21
		30	SD	0.1	30	30	30	27	26	21	21
		30	ST	0.1	29	29	29	27	22	21	21
		30	SP	0.1	30	27	27	24	21	20	19
		30	SA	0.1	30	25	23	21	14	11	11
		20	Control		0	0	0	0	0	0	0
CF3	580	30	SPZ	0.025	29	29	29	27	21	20	18
		29	SD	0.025	29	28	28	25	20	15	13
		30	ST	0.025	27	20	17	9	8	8	8
		30	SP	0.025	27	19	18	10	7	6	5
		29	SA	0.025	27	25	25	17	13	12	12
		20	Control		0	0	0	0	0	0	0
	130	30	SPZ	0.1	30	30	30	29	25	21	21
		30	SD	0.1	30	28	28	28	24	21	21
		30	ST	0.1	30	29	28	20	12	10	10
		30	SP	0.1	30	29	29	23	17	15	15
		30	SA	0.1	30	30	28	26	20	19	19
		20	Control		1	1	1	1	1	1	1
CF4	340	30	SPZ	0.025	28	28	28	23	17	16	16
		30	SD	0.025	30	30	30	26	20	20	19
		29	ST	0.025	27	25	24	17	8	3	3
		30	SP	0.025	30	30	28	16	8	3	2
		30	SA	0.025	28	25	22	11	7	5	2
		20	Control		0	0	0	0	0	0	0
	100	30	SPZ	0.1	30	30	30	27	20	19	19
		29	SD	0.1	29	29	29	28	25	22	21
		29	ST	0.1	29	26	24	15	3	3	3
		30	SP	0.1	30	30	29	16	9	9	9
		30	SA	0.1	30	28	28	15	8	6	1
		20	Control		3	2	0	0	0	0	0

TABLE 3—(*Concluded*)

INFECTING ORGANISM	NUMBER OF ORGANISMS IN INFECTING DOSE	NUMBER OF MICE INFECTED	TREATMENT		NUMBER OF SURVIVORS						
			Drug*	Dosage	Days after infection						
					1	2	3	5	10	20	30
				mgm.							
CF5	490	30	SPZ	0.025	29	29	29	26	21	20	16
		30	SD	0.025	30	30	30	30	24	20	12
		30	ST	0.025	28	25	21	13	10	10	10
		30	SP	0.025	30	29	27	22	18	18	18
		30	SA	0.025	29	27	24	19	15	14	13
		20	Control		1	0	0	0	0	0	0
	140	30	SPZ	0.1	30	30	30	30	28	28	26
		30	SD	0.1	30	30	30	30	27	25	25
		30	ST	0.1	30	30	30	23	21	20	18
		30	SP	0.1	30	30	29	26	24	22	15
		30	SA	0.1	30	30	30	30	27	22	20
		19	Control		3	1	0	0	0	0	0

* SPZ = sulfapyrazine; SD = sulfadiazine; ST = sulfathiazole; SP = sulfapyridine; SA = sulfanilamide.

2. Concentrations of free sulfonamide in the blood of mice following different doses of the various drugs. There were important differences in the concentrations of the different sulfonamides present in the blood 2, 4 and 6 hours after administration of single doses (table 4). At all dose levels, sulfadiazine gave the highest maximum concentrations. For example, after administration of 1.0 mgm. of the different sulfonamides, the maximum concentration of sulfadiazine was 7.9 mgm. % as compared with 3.4 mgm. % sulfapyrazine, 2.2 mgm. % sulfapyridine, 1.6 mgm. % sulfanilamide, and 1.2 mgm. % sulfathiazole. On the lower doses both sulfadiazine and sulfapyrazine gave considerably larger concentrations than were obtained with the other three sulfonamides. Following the administration of 0.2 mgm. doses, the maximum concentration of sulfapyrazine was 1.4 mgm. % and of sulfadiazine 2.2 mgm. %, whereas there was only 0.5 mgm. % of sulfapyridine and sulfanilamide present and somewhat less than that amount of sulfathiazole.

The concentrations of sulfapyrazine differed from those of the other sulfonamides in a number of respects. In the first place, the concentrations of this drug on the lowest dose were larger than those of any other drug except sulfadiazine. Thus 1.1 mgm. % sulfapyrazine was present in the blood 2 hours after administration of the 0.1 mgm. dose The concentration of sulfadiazine was similar to this, but only traces of the other compounds could be detected.

Sulfapyrazine also differed from the other drugs in that the concentrations in the blood increased to a lesser degree with large increases in dosage. Thus comparing the results with doses of 0.5 and 5 mgm., the maximum concentrations of sulfapyrazine were 2.8 and 5.2 mgm. % respectively. In contrast with this, the levels of sulfadiazine were 4.7 and 19.2 mgm. %; of sulfanilamide, 1.0 and

8.3 mgm. %; of sulfapyridine, 0.9 and 11.1 mgm. %, and of sulfathiazole, 0.8 and 5.1 mgm. %. It will be noted that the maximum concentration of sulfa-

TABLE 4

Concentrations of free sulfonamide in blood of mice receiving single doses of sulfapyrazine, sulfadiazine, sulfathiazole, sulfapyridine or sulfanilamide

DRUG	DOSE	MGM. PER CENT SULFONAMIDE IN BLOOD		
		Hours after administration		
		2	4	6
	mgm.			
Sulfapyrazine	0.025	trace*	trace	trace
	0.1	0.9	1.1	0.9
	0.2	1.4	1.4	1.4
	0.5	2.8	2.1	2.2
	1.0	3.4	3.0	2.9
	2.5	3.8	3.5	3.0
	5.0	4.7	4.8	5.2
Sulfadiazine	0.025	trace	trace	trace
	0.1	1.3	1.3	0.7
	0.2	2.2	1.8	1.3
	0.5	4.7	4.0	3.0
	1.0	7.9	6.8	4.3
	2.5	13.4	11.4	9.5
	5.0	19.2	14.7	11.4
Sulfathiazole	0.1	trace	trace	trace
	0.2	trace	trace	trace
	0.5	0.8	trace	trace
	1.0	1.2	trace	trace
	2.5	2.8	1.3	0.8
	5.0	5.1	4.1	1.3
Sulfapyridine	0.1	trace	trace	trace
	0.2	0.5	trace	trace
	0.5	0.9	0.5	trace
	1.0	2.2	1.0	0.8
	2.5	4.8	3.3	1.4
	5.0	11.1	9.5	5.7
Sulfanilamide	0.1	trace	trace	trace
	0.2	0.5	trace	trace
	0.5	1.0	trace	trace
	1.0	1.6	0.7	trace
	2.5	3.1	1.3	0.8
	5.0	8.3	3.1	1.6

* Detectable but less than 0.5 mgm. %.

pyrazine on the largest dose (5 mgm.) was lower than that of any other sulfonamide excepting sulfathiazole—5.2 mgm. % sulfapyrazine as compared with 8.3,

11.1 and 19.2 mgm. % of sulfanilamide, sulfapyridine and sulfadiazine. Since sulfathiazole is absorbed and excreted very rapidly and the maximum concentration occurs in the blood prior to 2 hours, it seems probable that the peak concentration of this sulfonamide was also greater than that of sulfapyrazine.

A third difference was that the concentrations of sulfapyrazine in the blood were maintained at more uniform levels than those of the other sulfonamides. Regardless of dosage, the concentrations of sulfapyrazine present at 6 hours were nearly identical with those present at 2 and 4 hours. On the other hand, the concentrations of the other sulfonamides were usually much lower at 4 and 6 hours, the decrease being less marked with sulfadiazine than with the other drugs.

DISCUSSION. The present study has shown that sulfapyrazine is a highly effective drug against infections with *beta* hemolytic streptococci in mice. Dose for dose it compares favorably with sulfadiazine in activity and is distinctly superior to either sulfanilamide, sulfapyridine or sulfathiazole. This order of activity of the different sulfonamides was the same against infections with all strains and at all dosages.

Much of the effectiveness of sulfapyrazine, and of sulfadiazine as well, depends upon absorption and excretion characteristics that make it possible to maintain effective concentrations in the blood during the entire period between treatments. These characteristics are not common to the other sulfonamides, particularly sulfanilamide and sulfathiazole, which are absorbed and excreted so rapidly that effective concentrations are maintained for only short periods after the administration of small doses.

That the superiority of sulfapyrazine and sulfadiazine over the other sulfonamides in experimental streptococcal infections is due largely to these characteristics is shown by a comparison of the activities of the different drugs when the average concentrations maintained in the blood were similar. Such a comparison has been made by selecting experiments on strain C203 (table 2) in which the dosage of drug (according to the data in table 4) would have been expected to give average sulfonamide concentrations in the blood ranging from 1.0 to 1.3 mgm. % and from 3.1 to 4.1 mgm. %.[5] This comparison (table 5) shows clearly that, in either concentration range, the activities of sulfapyrazine, sulfadiazine, sulfathiazole and sulfapyridine were essentially equal. It should be noted that the doses of sulfapyridine, sulfathiazole and sulfanilamide required to give the lower range of concentrations were 10 to 25 times the doses of sulfapyrazine and sulfadiazine. In the higher concentration range, the activity of sulfanilamide was also identical with that of the other drugs. In the lower range, sulfanilamide was slightly more active. This slight difference may have been without significance in view of the crudeness of the comparison. On the other hand, it may have been due to the fact that the maximum concentration of sulfanilamide

[5] The concentrations regarded as average were those that prevailed 4 hours after administration of the respective drugs. Blood levels obtained at this time were selected as more nearly representative of the average concentrations present in the blood during the 8 hour intervals between treatments than the levels present at any other period.

present was much higher than that of any other drug except sulfathiazole. Maximum concentrations as well as the average or the minimum concentrations undoubtedly influence therapeutic activity.

Since in both streptococcal and pneumococcal infections the activity of sulfapyrazine is only equivalent to that of equal doses of sulfadiazine, and its toxicity is essentially identical with that of sulfadiazine (3, 6), the question may be raised whether this new sulfonamide derivative merits any place in the therapy of bacterial infections. The final answer to this question will have to await a careful evaluation of the clinical effectiveness of the two drugs. However, it may be predicted that sulfapyrazine will be a useful addition, because it has a very high bacteriostatic activity against a variety of organisms (5, 11, 12) and its slow absorption and excretion make possible the maintenance of uniformly

TABLE 5

Comparative activity of the sulfonamides when similar concentrations are maintained in the blood

(Data selected from tables 2 and 4)

DRUG	DOSE	MCM.%* IN BLOOD	PER CENT 30-DAY SURVIVORS
	mgm.		
Sulfapyrazine	0.1	1.1	40
Sulfadiazine	0.1	1.3	43
Sulfathiazole	2.5	1.3	40
Sulfapyridine	1.0	1.0	33
Sulfanilamide	2.5	1.3	63
Sulfapyrazine	2.5	3.5	73
Sulfadiazine	0.5	4.0	70
Sulfathiazole	5.0	4.1	60
Sulfapyridine	2.5	3.3	57
Sulfanilamide	5.0	3.1	73

* Concentration 4 hours after administration of sulfonamide.

effective concentrations in the blood for long periods even when small doses are administered. Furthermore, the fact that increasing the oral dosage of sulfapyrazine produces smaller increases in the concentration in the blood than are produced by the other sulfonamides should give sulfapyrazine a margin of safety when these drugs must be used clinically without close control of the blood levels. This failure to produce high concentrations can also be a disadvantage in sulfapyrazine when such concentrations are essential for a good therapeutic response. However, the work of Hamburger and colleagues (13) has shown that this disadvantage may be offset by intravenous therapy with sodium sulfapyrazine.

It should also be pointed out that the slow absorption of sulfapyrazine and its consequent retention in considerable quantities in the intestinal tract should make this drug useful in the treatment of intestinal infections. This has already been indicated by the experimental work of White (11) on the reduction in

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After control observations were recorded, various narcotics were administered and their effects noted on the two factors mentioned above. Chloralose, nembutal, sodium barbital, and sodium pentothal were given intravenously; morphine sulfate was injected intramuscularly and ether and cyclopropane were administered by inhalation. An ether-air mixture was inhaled directly into the tracheal cannula from a vaporizer. An increase in the concentration of the vapor could be obtained by shunting more of the inspired air over the liquid ether. Inhalations lasted for 15–30 minutes. Cyclopropane (25% in O_2) was administered by an open technique, with water valves, 75 liters of the gas mixture in a Douglas bag being attached to the inspiratory side. Observations were made at the end of a 30 minute period of inhalation. Central response was measured by a mixture of 10% CO_2, 67.5% O_2, and 22.5% cyclopropane to avoid changing the depth of anesthesia.

RESULTS. The one common and consistent result with the 7 drugs studied was depression of the respiratory response to the inhalation of CO_2. There was a definite relationship between degree of depression and depth of narcosis, until, in very deep narcosis in many experiments, CO_2 produced either no change at all in breathing, or was depressant.

In addition, with the exception of ether, all of the drugs decreased the minute volume of breathing. Again there was a correlation with depth of narcosis, although this was less striking than that noted with the CO_2 response. Inhalation of ether increased minute volume in both the cat and the dog.

Great variability was noted in the effect of narcosis on the sensitivity of the chemoreceptors. Their results will be discussed as the individual drugs are presented.[2]

Chloralose. Seven dogs were studied. Figure 1 illustrates a typical response. After chloralose, the central response to CO_2 was considerably diminished. At the same time the sensitivity of the chemoreceptors to NaCN had increased, for a dose which had no effect before now increased breathing distinctly. Similar results were obtained in all of the 7 experiments of the sort (fig. 2).

Chloralose thus is seen to cause a shift in the balance of these two factors involved in respiratory control in the dog so that the part played by direct chemical stimulation of the center is diminished and that played by the chemoreceptor reflexes is augmented—a phenomenon first described by Marshall and Rosenfeld in 1936 (6). Experiments on dogs anesthetized with chloralose therefore give an exaggerated idea of the importance of these reflexes and that fact should be borne in mind in interpreting the results. This has been confirmed by Watt *et al.* (7) in normal, unanesthetized dogs.

[2] Control experiments were conducted in 4 decerebrated dogs and in 6 decerebrated cats, in which observations of resting pulmonary ventilation, of the response to CO_2 inhalation, and of the effectiveness of NaCN, were made at 15 minute intervals over a period of 2 hours without the administration of a narcotic drug In the cats no significant changes were seen. In all of the dogs there was a tendency toward an increase in respiratory minute volume and in the response to CO_2 while the threshold dose of NaCN either remained unchanged or increased slightly. These control experiments indicate that decrease in pulmonary ventilation and in the response to CO_2, and increased or markedly decreased effectiveness of NaCN, associated with the administration of narcotic drugs, can properly be ascribed to the drugs and not to coincidental changes in the condition of the animals.

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When chloralose was given to decerebrated cats, we realized for the first time that we had to deal with *species differences* as an additional complicating factor. In the 9 cats used, chloralose produced no change in the sensitivity of the chemoreceptors, *the dose of NaCN which was threshold during the control period* being just as effective as narcosis progressed. Quantitatively, cats also differed from dogs in showing a greater depression of minute volume and a more marked decrease in reactivity to CO_2 (fig. 2).

Morphine. Following the intramuscular administration of increasing amounts of morphine sulfate to 5 dogs and 8 cats, results similar to those obtained with chloralose were noted. Certain quantitative species differences deserve men-

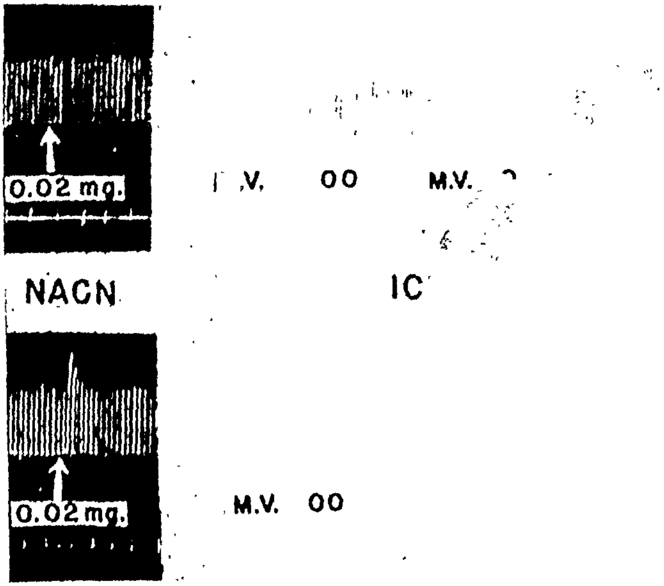
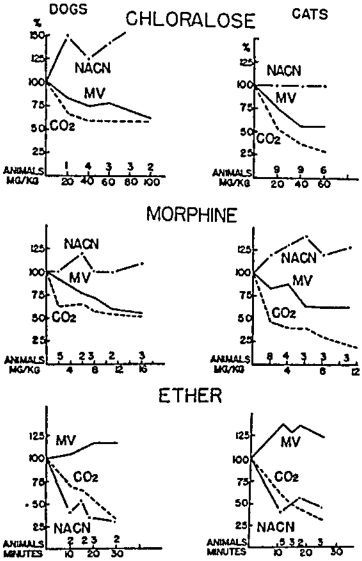


FIG. 1. EFFECT OF CHLORALOSE
Upper tracings taken during control period; tion of chloralose 40 mgm./kgm. Pneumograph each stroke being equal to 500 cc.

tion. The increased sensitivity of the c dent in cats than in dogs. Furthermore, to a greater extent in cats (fig. 2).

Barbituric acid derivatives. A long-, member of this series were studied, i.e., and pentothal sodium. All these dru minute volume and in the response to C sensitivity of the chemoreceptors was tere Definite and m ted in b

was given to 4 dogs and 8 cats. In the dogs there was no change in chemoreceptor reactivity, whereas the cats showed a progressive increase in this reactivity as anesthesia deepened. *Pentothal,* in 4 dogs and 6 cats, did little as far as chemoreceptor thresholds were concerned.



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Ether. A pattern entirely different from those already described emerged from the investigation of this agent in 4 dogs and 5 cats. As has been mentioned before this was the only narcotic studied which stimulated respiration, the respiratory minute volume being greater than during the control period. The central response to CO_2 inhalation was progressively decreased as anesthesia continued. The sensitivity of the chemoreceptors was depressed markedly and consistently. All of these effects were noted in both species of animals (fig. 2).

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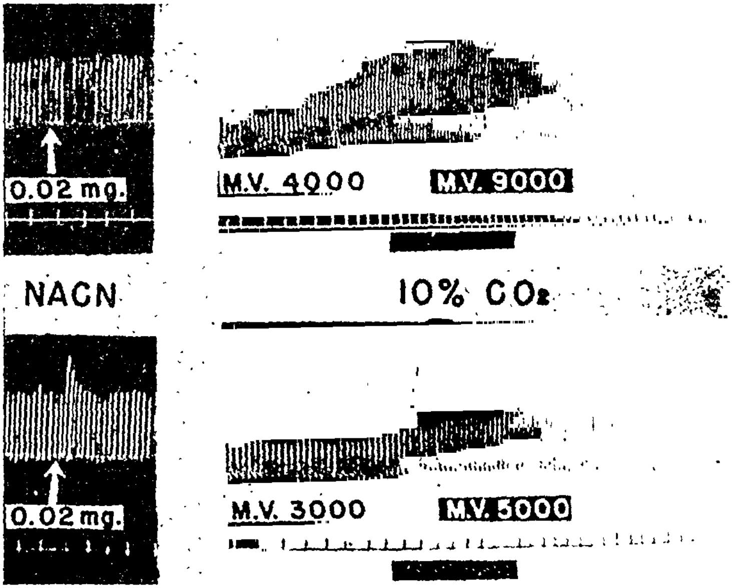
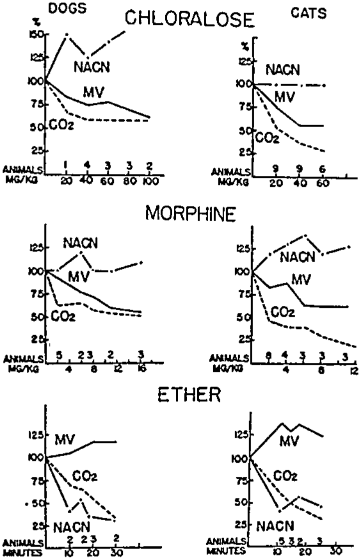


Fig. 1. Effect of Chloralose on Respiration of Dog
Upper tracings taken during control period; lower tracings taken after the administration of chloralose 40 mgm./kgm. Pneumograph record at top—minute volume at bottom, each stroke being equal to 500 cc.

tion. The increased sensitivity of the chemoreceptors to NaCN was more evident in cats than in dogs. Furthermore, morphine decreased the CO_2 response to a greater extent in cats (fig. 2).

Barbituric acid derivatives. A long-, an intermediate-, and a short-acting member of this series were studied, i.e., barbital sodium, pentobarbital sodium, and pentothal sodium. All these drugs produced reduction in respiratory minute volume and in the response to CO_2 inhalation. The influence on the sensitivity of the chemoreceptors was variable (fig. 3). *Barbital* was administered to 5 dogs and 7 cats. Definite and consistent sensitization of the aortic and carotid chemoreceptors resulted in both species of animals. *Pentobarbital*

was given to 4 dogs and 8 cats. In the dogs there was no change in chemoreceptor reactivity, whereas the cats showed a progressive increase in this reactivity as anesthesia deepened. *Pentothal,* in 4 dogs and 6 cats, did little as far as chemoreceptor thresholds were concerned.



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Cyclopropane. This anesthetic was only administered to cats and the dat represent only a preliminary report. In the four animals studied marked de pression of minute volume, CO_2 response and chemoreceptor sensitivity wer observed (fig. 4). This is the only one of the drugs studied which depressed a three of these functions.

A partial explanation for the scattered results found in this study of the chem oreceptors was obtained when a set of control experiments was undertaken t

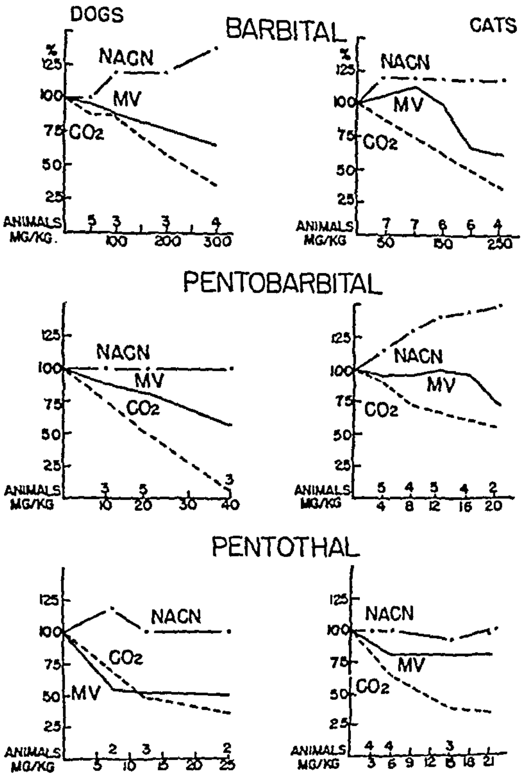

FIG. 3. See legend for fig. 2

determine how much of the effect of cyclopropane in cats was due to the narcotic and how much to the 75% oxygen with which it was given. The results showed that when the oxygen content of the inspired air was high, more NaCN was required to stimulate the chemoreceptors and the resulting increase in minute volume was less than had been the case during air breathing. Conversely, when the inspired mixture contained less oxygen than room air the response to cyanide was increased and the threshold lowered (fig. 5). Since the threshold dose of NaCN was so dependent upon oxygen tension, it became of interest to determine

whether other substances which stimulate the chemoreceptors were affected in like manner. Accordingly alpha-lobeline was investigated. It was found that the threshold dose of alpha-lobeline was not changed by variations in oxygen tension (fig. 5). This supports the belief of von Euler, Liljestrand, and Zotter-

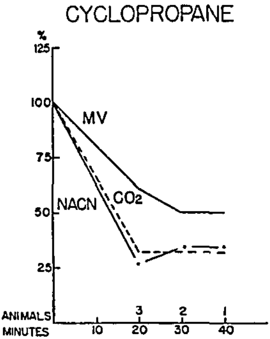

FIG. 4

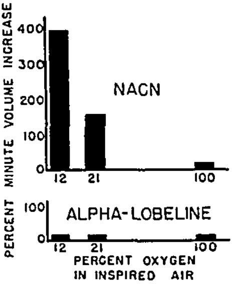

FIG. 5

FIG. 4. See legend for fig. 2

FIG. 5. INFLUENCE OF OXYGEN ON THE RESPIRATORY RESPONSE TO INTRAVENOUS INJECTION OF NaCN AND ALPHA-LOBELINE

The same dose of each drug was used throughout

man (8) that ganglion cells are present in the region of the carotid and aortic bodies. Presumably NaCN affects end organs which are activated by oxygen lack, whereas lobeline acts upon ganglion cells in a "nicotinic" fashion. The latter cells are apparently not affected by changes in oxygen tension.

When the data were re-examined with this in mind, it was found that in most cases in which the chemoreceptor reflexes were exaggerated, there was considerable depression of breathing, and anoxemia was probably present. A few actual determinations of arterial oxygen tensions showed this to be the case. For example, under morphine anesthesia (11 mg. per kg.), one dog's oxygen tension was reduced from a control level of 83 mm. Hg to 54 mm. Hg. In another dog under ether (which depressed chemoreceptor activity), oxygen tension rose from 70 mm. Hg in the control period to 75 mm. Hg under anesthesia, minute volume increasing from 4000 cc. to 5000 cc.

It seems probable therefore that the sensitization of the chemoreceptors—evident in reduction in the size of the minimum effective dose of NaCN—is due largely if not entirely to the presence of anoxemia by which the chemoreceptor reflexes are already stimulated and with which the NaCN summates. Additional evidence in the same direction was obtained when these animals were

made to breathe 100% oxygen, for we found that prolonged depression of breathing took place only when considerable anoxemia had been present. Watt *et al.* (7) have shown that oxygen inhalation leads to a temporary depression of minute volume even in normal, unanesthetized dogs, hence, only sustained decreases are significant as indices of an increasing chemoreceptor activity. The results of our experiments do not permit us to agree with Moyer and Beecher (9) who state that the shift to "control by chemosensitive reflex mechanisms activated by anoxia, is in many instances unattended by changes in the rate or character of breathing." It has been our experience that such a shift is accompanied by a decrease in minute volume leading to underventilation and anoxemia.

Whether this difference is due to our use of decerebrated preparations we are not at present prepared to say. Our reason for selecting decerebrated animals was that we were thus enabled to start with essentially an unanesthetized animal.

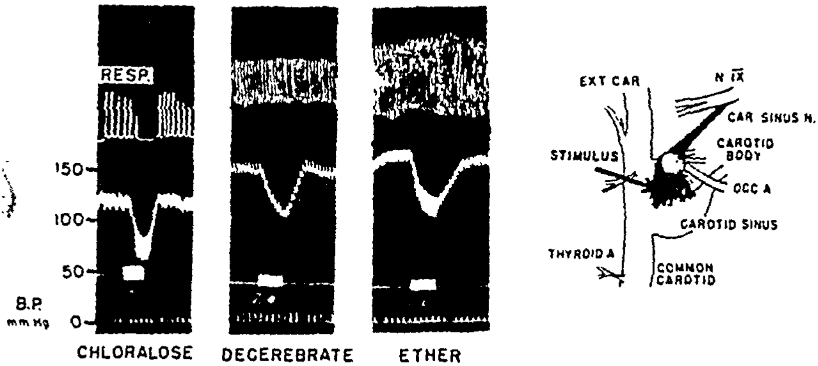

FIG. 6. FARADIC STIMULATION OF CAROTID SINUS PRESSURE FIBRES OF THE DOG
From above downward, pneumograph record of respiration, femoral artery blood pressure, strength of electrical stimulus (Harvard inductorium), time in 5 sec.

We realize that severance of the brain stem alters physiological responses in a number of ways, and it is difficult to know how far such alterations may go. One instance of interest was illustrated when a study of the effect of narcotics on the carotid sinus pressure receptors was undertaken. Under chloralose anesthesia, electrical stimulation of the depressor fibres of the carotid sinus nerve with a faradic current leads to a fall in blood pressure, bradycardia, and apnea. Under ether anesthesia a similar stimulation reproduces the entire circulatory picture, but apena does not occur (fig. 6). Decerebration has the same effect as ether; the cardiovascular effects are unchanged but apnea does not occur. This similarity between decerebration and ether anesthesia is interesting in connection with certain observations which others have made. For example, Heinbecker and Bartley (10) stimulated the isolated saphenous nerve, recording action potentials in the appropriate area of the sensorimotor cortex of the cat. Under ether anesthesia transmission of impulses from the periphery was de-

creased "at a time when spontaneously active elements in the cortex are little affected indicating that afferent fibre pathways below the cortex are quickly blocked." Furthermore, Meek and his co-workers (11) have shown that decerebration prevents the onset of cardiac arrhythmias which are frequently noted during cyclopropane anesthesia in dogs. Ether likewise prevents these irregularities which are thought to be due to impulses travelling down sympathetic pathways from the hypothalamus. Here is another illustration of the blocking effect which ether appears to exert on the transmission of nervous impulses.

Discussion. In an earlier communication from this laboratory (4) the results of some of these experiments were briefly reported and an attempt was made at evaluation and explanation in terms of the information then available. Additional experiments with the drugs discussed in that report, as well as with certain others, have enabled us to confirm the earlier interpretations in all essential respects but one: we now believe that exaggeration of chemoreflex functions by certain narcotics can be produced simply by depression of the center's ability to respond directly to carbon dioxide and does not necessarily require an actual increase in reflex excitability, as was suggested in the earlier publication. The change is necessitated by the subsequent findings that barbital (which does not increase reflexes) behaves in this regard like chloralose and morphine (which do,) and that chloralose does not exaggerate chemoreflexes in the cat although it causes increased reflex excitability. The additional finding that removal of anoxemia (by inhalation of oxygen) may entirely remove this exaggeration of chemoreflex excitability indicates that the change is probably only an apparent one and is due to the presence of an abnormal amount of chemoreceptor activity aroused by anoxemia resulting from the diminution in pulmonary ventilation. The further slight changes in chemoreceptor activity produced by intravascular injections of cyanide probably are more effective under such circumstances because the activity of a large number of receptors is involved and the effects on the respiratory center should be correspondingly greater.

Apart from this, we have no new data bearing on the explanation for this transformation in respiratory control from the normal pattern, in which exquisite sensitivity of the center to carbon dioxide is the outstanding feature and chemoreceptor reflexes are unimportant if present at all, to that elicited by narcotic drugs in general, in which the responsivity of the center to carbon dioxide decreases even to the point of complete disappearance while chemoreceptor reflexes may be relatively increased, unchanged, or (in the case of ether or cyclopropane) greatly diminished. A phylogenetic explanation for the relative resistance of the chemoreceptor system to depression by narcotics was presented in the earlier report (4), but we did not then and do not now have any corresponding explanation for the depression of this reflex system by ether and cyclopropane, alone among the drugs tried. It should be pointed out that the decreased reactivity to cyanide under the influence of cyclopropane was actually due to the cyclopropane and not to the oxygen with which it was given because the control observations with cyclopropane were made while the animals were

breathing 100% oxygen. Perhaps cyclopropane blocks the transmission of impulses in the central nervous system much as ether is known to do (10). The difference in this respect between these drugs and the barbiturates is probably not merely a quantitative one because with the latter there were no rises in chemoreceptor threshold comparable with those produced by ether or cyclopropane, even when the barbiturates were given to the limit of tolerance, i.e., to the point of marked respiratory depression and profound muscular relaxation. Further investigation along these lines seems desirable and we propose to undertake it at some more propitious time.

The one constant respiratory effect that was common to all the drugs was depression of the response to inhalation of carbon dioxide. This probably is another manifestation of the well-known "descending depression" produced by narcotics, and this, in turn, appears to be referable to the complexity of the system acted upon. According to Winterstein (12), one of the outstanding features in the action of narcotics on living structures is the direct relationship between the degree of differentiation or specialization and susceptibility to depression by these drugs. The great sensitivity of the normal mammalian respiratory center to carbon dioxide is peculiar to this class of animals and may be regarded as a recent acquisition as well as a highly specialized one. Response to afferent impulses from the chemoreceptors, on the other hand, appears to be survival of the more primitive type or respiratory control seen in aquatic animals (6) (4) (3) and should therefore involve a simpler system. Perhaps the impulses from these structures also are capable of producing a more marked central disturbance than any of the other influences concerned in respiratory control. This is supported by the ability of chemoreceptor denervation to prevent the convulsions as well as the hyperpnea, tachycardia and hypertension seen after the intravenous injection of cyanide in unanesthetized dogs (13). The peculiar ability of ether to raise the chemoreceptor thresholds may be another manifestation of its outstanding ability to block transmission in the central nervous system. It is a remarkable fact that ether, which is the only one (except cyclopropane) among the drugs used that consistently depressed both the central response to CO_2 and the chemoreceptor response to cyanide, was still the only drug that did not depress respiratory minute volume. This may mean, as Henderson and Rice (14) have suggested, that ether directly stimulates the respiratory center to a conspicuous extent. This explanation has recently gained in authority by Krogh's statement (15): "The general effect of CO_2 is somewhat similar to that of a narcotic, stimulating within certain narrow limits and above that depressing." Ether is also the most likely of all surgical anesthetics to set up reflexes by local irritation both in the respiratory tract and elsewhere, because in the concentrations employed for anesthesia it is the most irritant of anesthetics. Recent work (16, 17) has shown that reflexes probably play a more important part in respiratory control than has been generally realized. Some evidence has recently been obtained (16) to indicate that ether, perfused through a hindlimb in anesthetic concentration, can stimulate breathing reflexly. This, in addition, to its well-known ability to arouse stimulant reflexes in the respiratory passages

(18, 4, 14), offers an alternative explanation for the absence of respiratory depression by ether even though the central response to CO_2 and the chemoreceptor response to cyanide are both markedly depressed.

These experiments are in agreement with those of Marshall and Rosenfeld (6) and Beecher and Moyer (19, 9). The latter have also studied the effect of certain anesthetics on vago-pulmonary respiratory reflexes (20, 21, 22). From all of these data it is evident that no single pattern can be described for the effect of anesthetics on the control of respiration. This is a complex resultant of many forces. It should be pointed out, however, that because of differences in drug action and species response, conclusions based on a study of one substance in one species of animal can not be transferred directly to a consideration of narcosis and respiratory control in man.

CONCLUSIONS

1. Chloralose, ether, morphine, barbital, pentobarbital, and pentothal have been administered to decerebrate dogs and cats, cyclopropane to decerebrate cats. The effect of these anesthetics on respiratory minute volume, respiratory response to inhalation of CO_2, and sensitivity of the chemoreceptors of the carotid and aortic bodies has been measured.

2. There were considerable variations among the various drugs and in the species response to these drugs. Only one finding was constant for all the drugs and both species of animals, namely, a progressive decrease in respiratory response to CO_2. Next to this in constancy was the depression of respiratory volume which occurred with all of the drugs but ether.

3. The effect of the anesthetics on chemoreceptor activity can be divided into two groups. The first, including ether and cyclopropane, decreased chemoreceptor sensitivity. All the other drugs either did not change the sensitivity significantly or actually increased it. This increase in sensitivity to cyanide is probably due, at least to some extent, to the presence of anoxemia resulting from respiratory depression, because removal of anoxemia by inhalation of O_2 restores the sensitivity approximately to normal. The sensitivity to alpha-lobeline is not altered by O_2 inhalation which suggests that the receptors acted upon by this drug are not the same as those affected by anoxemia or cyanide.

4. Some of the possible implications of these findings to respiratory control under anesthesia are discussed.

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STUDIES CONCERNING THE LUTEOID ACTION OF STEROID HORMONES

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It is well known that several steroids share with progesterone the ability to imitate the actions of a functional corpus luteum. Yet systematic studies concerning the correlation between the luteoid and other pharmacological effects, or the chemical structure of the steroids, have not been undertaken. The majority of the relevant data published up to the present are to be found in footnotes and remarks attached to the papers of chemists who synthesized these steroids and then assayed them mostly on one or two animals only. On the other hand the few biologists who showed some interest in pharmaco-chemical correlative studies apparently did not appreciate the necessity for identifying their compounds by systematic chemical names and physical constants (M.P., optic rotation) indicating the degree of purity of the samples used. Thus terms such as "androstenediol" have commonly been employed although it is evident that this designation would apply to a vast number of compounds irrespective of the position of the double bond and of the two hydroxyl groups. It is well to remember, furthermore, that these compounds are either prepared from tissues and body fluids in which they occur in mixtures, or by partial synthesis from other steroids. In the former case contamination with other hormone-like substances, and in the latter with unchanged parent compounds, is likely to interfere with pharmacological assays. Not only can such contaminations produce false positives due to the luteoid effect of the contaminant, but if the latter possesses strong folliculoid properties, traces of contamination may suffice to mask the luteoid action of the compound tested. Another severe drawback of the relevant published results, concerning all but a few of the most active compounds, is that assays were performed on animals of uneven weight and frequently on one dose level only. Although most of the assays were done on rabbits, different technics have been used by various investigators, and sometimes even by the same investigator, since a rather expensive large rabbit colony must be maintained to assure a constant adequate supply of animals of a specified size and age.

In order to obtain a set of precise inter-comparable data concerning pharmacological and pharmaco-chemical correlations between various luteoid compounds, our bioassays were performed on more than 200 rabbits of the same colony; they weighed 800–1100 g. with an average very close to 900 g. Forty-five steroid compounds were thus assayed on the intact immature female according to the technic of McPhail (1) with the only modification of using three subcutaneous injections of 5 γ of estradiol in 0.1 cc. of peanut oil subcutaneously every second day during the period of sensitization. In agreement with most other investi-

gators who used the progestational activity as an indicator of luteoid properties, we considered the minimum amount necessary to produce a ++ to +++ response (McPhail's scale) as equivalent to 1 rabbit unit (Rb.U.). It will be noted that our unit is somewhat smaller than that of most investigators. This may either be due to our technic of sensitization or to the great uniformity of the animal material used. Thus 250 γ of progesterone or $\frac{1}{4}$ of an international unit (I.U.) corresponded to 1 Rb.U. in our test.

The results of our observations are summarized in table 1. Each compound is designated by its full systematic chemical name (for terminology see Selye (2)) as well as (in italic type) by its popular name if such is commonly in use. The steroids are arranged according to their chemical structure enumerating estrane, androstane, alkyl-substituted androstane, etiocholane and alkyl-substituted etiocholane derivatives in this order. Within each of these groups the individual compounds are arranged according to increasing numbers of carbon atoms; these being equal, the arrangement is according to increasing degrees of unsaturation; and where this is equal, according to increasing degrees of oxygenation. An asterisk underneath the serial number of the compound indicates that it is a natural compound inasmuch as its presence in animal tissue has been demonstrated. The esters of natural compounds are marked "E." The name of the chemist who supplied us with the compound is also mentioned. Since the ucture of some steroids has not been completely proven as yet, the melting int of the sample used was determined in our laboratory and is given in order avoid any uncertainty concerning the identity of the product and the purity of the sample used. Beneath the melting point of our preparation, that given in the literature for highly purified specimens is recorded in parentheses for comparative purposes. In the case of compounds not previously described, this could not be done of course. It will be noted that most of our samples were highly purified and some were purer than the best preparations described up to the present time. All steroids were administered in 0.2 cc. of peanut oil per injection. The total dose was administered in five daily subcutaneous injections and it is the total amount given per animal that is registered in our table for each dose level. In the next column we give the number of animals used on a certain dose and after that the response according to McPhail's scale. If several animals on the same dose level responded differently the number giving the same response is indicated in brackets. In two instances the compound was directly injected into the uterine cavity to detect traces of activity. However, this technic proved (on the basis of experiences not published here) to be only of qualitative value. Although in some cases there was not sufficient material available to determine the degree of activity with accuracy, we expressed the luteoid potency of each compound in I.U. as closely as our data permitted. It will be recalled that by definition 1 I.U. equals the activity of 1 mgm. of crystalline progesterone (3). In the last column we recorded the literature concerning the luteoid activity of the compounds which had received attention from previous investigators. These data are roughly classified according to the results obtained into inactive, slightly active, medium active and highly

TABLE 1

Luteoid action of steroid hormones

NO.	NAME	M. P.	DOSES	NO. OF ANIMALS	RESPONSE	MG./I.U.	REFERENCES
		°C.	mg.				
1 *	$\Delta^{1,3,5}$-Estratriene-3,17(α)-diol, *α-estradiol* (Schwenk)	176 (176-177)	0.500 0.030 0.015	1 1 1	0 0 0	0	in : 2
2	Androstane (Heard)	48.5-49.5 (49-50)	50	1	0	0	
3	*Androstane-17(α)-ol, androstanol* (Heard)	167-168 (166)	20	1	++	80	
4 *	Androstene-3-(α)-ol.-17-one, *androsterone, cis-androsterone* (Hoffman)	178-180 (180-181)	44	1	0	0	in.: 4, 5
5	Androstane-3,17-dione (Heard)	132-134 (132-133)	20	1	+	>80	
6 *	Δ^4-Androstene-3-one-17 (α)-ol, *testosterone* (Schwenk)	154 (154-154.5)	1 10 20 35 50	1 4 4 3 1	0 0 0 0 + ++ (1) (1) (1) +++	140-200	sl.: 6; med.: 4
7 E*	Δ^4-Androstene-3-one-17(α)-ol, propionate, *testosterone propionate* (Schwenk)	118 (121)	5 10 12.5 25	4 3 3 2	0 + (2) (2) 0 + (2) (1) ++ +++ (1) (2) ++ +++ (1) (1)	50	med : 7-10
8	Δ^4-Androstene-3,17-dione, *androstenedione* (Holden)	170 (169-174)	25 35 50	3 3 1	0 + ++ (1) (1) (1) + ++ (1) (2) +++	140-200	in : 6, 7, 11, 12; sl.: 4
9	Δ^4-Androstene-3,17-dione-6(α)-ol acetate (Ehrenstein)	176 (176)	4.5	2	0	0	
10	Δ^4-Androstene-3(β),17(β)-diol (Holden)	203-204 (198-198.5)	50	3	0	0	
11	Δ^4-Androstene-3(β),17(β)-diol-17-propionate (Holden)	140	50	3	0	0	
12	Δ^4-Androstene-3(β),17(β)-diol-3,17-dipropionate (Holden)	97	50	3	0	0	
13	Δ^4-Androstene-3(β),17(α)-diol (Holden)	184-165 (182-183)	50	3	0 + (1) (2)	>200	in.: 4; sl.: 6
14	Δ^4-Androstene-3(β),17(α)-diol-3,17-*dipropionate* (Holden)	119-120	50	3	0	0	

TABLE 1—*Continued*

NO.	NAME	M.P.	DOSES	NO. OF ANI-MALS	RESPONSE	MG./I.U.	REFERENCES
		°C.	mg.				
15 •	Δ^5-Androstene-3(β)-ol-17-one, *dehydro-iso-androsterone, trans-dehydroandrosterone* (Schwenk)	148 (144–153)	50	3	0	0	in.: 4, 6, 8
16 E•	Δ^5-Androstene-3(β)-ol-17-one acetate, *dehydro-iso-androsterone acetate* (Schwenk)	169 (169)	50	3	0	0	
17	Δ^5-Androstene-3-chloro-17-one, *3-chloro-dehydro-iso-androsterone* (Fieser)	155–156 (155–156)	52	1	0	0	
18	17-Methyl-Δ^4-Androstene-3-one-17-ol, *methyl testosterone* (Schwenk)	163 (163–164)	1 7.5 10 50	1 3 3 1	0 + ++ (1) (2) ++ ++++ (2) (1) +++	30	med.: 4, 6
19 •	17-Ethyl-androstane-3(β),21-diol-11,20-dione, *Kendall's CPD. "H"* (Kendall)	139 (189–191)	2† 20	1 1	0 0	0	
	17-Ethyl-Δ^4-androstene-3,20-dione, *progesterone* (Schwenk)	128 (128)	0.2 0.25 0.5 1 10	4 4 3 1 1	0 + (2) (2) ++ +++ (3) (1) +++ ++++ (1) (2) ++++ ++++	1	High: 5, 13 14
21	17-Ethyl-Δ^4-androstene-3,20-dione-6(α)-ol-acetate, *6-hydroxy-progesterone acetate* (Ehrenstein)	145–146 (145–146)	2.5 5	2 1	0 ±	?	sl.: 15
22 E•	17-Ethyl-Δ^4-androstene-3,20-dione-21-ol acetate, *desoxycorticosterone acetate* (Schwenk)	152 (158–160)	1 2 2.5 3.75 5 7.5	4 3 4 3 5 3	0 + ++ (1) (2) (1) + (3) + ++ +++ (1) (1) (2) ++ +++ (2) (1) + ++ ++++ (2) (2) (1) +++ (3)	10	high: 16–19
23 •	17-Ethyl-Δ^4-androstene-3,11,20-trione-21-ol, *dehydro-corticosterone* (Kendall)	173 (174–180)	6	1	0	0	
24 •	17-Ethyl-Δ^4-androstene-3,11,20-trione-17,21-diol, *Kendall's CPD. "E"* (Kendall)	215–218 dec (215–218 dec)	2 8	4 1	0 ++++ (3) (1) 0	?	in.: 20

TABLE 1—*Continued*

NO.	NAME	M.P.	DOSES	NO. OF ANIMALS	RESPONSE	MG./I.U	REFERENCES
		°C.	mg.				
25	17-Ethyl-Δ⁵-androstene-3(β)-ol-20-one, *Δ⁵-pregnenolone* (Schwenk)	186 (186-187)	10 25 50	2 7 4	+ +++ (1) (1) 0 + +++ ++++ (1) (2) (3) (1) 0 ++ +++ ++++ (1) (1) (1) (1)	40-100	in.: 21, 22
26	17-Ethyl-Δ⁵-androstene-3(β),21-diol-20-one-21 acetate, *acetoxypregnenolone* (Schwenk)	183-184 (183-184)	1 5 25 50	1 2 3 4	0 0 0 + (1) (2) 0 + +++ (1) (2) (1)	>200	
27	17-Ethyl-Δ⁴,¹⁶-androstene-3,20-dione, *16-dehydroprogesterone* (Ruzicka)	186-187 (186-188)	25	1	+ to ++	>100	in.: 23
28	17-Ethyl-Δ⁵,¹⁶-androstadiene-3(β)-ol-20-one acetate, *Δ⁵,¹⁶-pregnadienolone acetate* (Kamm)	174-175 (176)	50	3	0	0	
29	17-Vinyl-Δ⁴-androstene-3-one-17-ol, *vinyl-testosterone* (Scholz)	139 (140-141)	5	1	+++	<20	high: 24
30	17-Ethinyl-Δ⁴-androstene-3-one-17(α)-ol, *ethinyl-testosterone* (Schwenk)	265-268 (270-272)	1 2.5 5	3 7 1	0 0 + ++ +++ (1) (1) (3) (2) +++	10	high: 24-26
31	17-Butyl-Δ⁴-androstene-3,20-dione, *21-ethyl-progesterone* (Ruzicka)	115 (118-120)	1 2† 5 10	1 3 1 1	++ ++ (3) 0 ++	4-40	in.: 27
32	17-Iso-heptyl-Δ⁴-androstene-3,25-dione, *Δ⁴-nor-cholestene-3,25-dione* (Schwenk)	114-116 (128-129)	1 10 50	1 1 3	0 0 0	0	
33	17-Iso-heptyl-Δ⁵-androstene-3(β)-ol-25-one, *Δ⁵-nor-cholestenolone* (Schwenk)	127-128 (125-127)	1 10 50	1 1 3	0 0 0	0	in.: 28
34 *	17-Iso-octyl-Δ⁵-androstene-3(β)-ol, *cholesterol* Merck & Co.	149 (150)	10 50	1 3	0 0	0	
35	*Diosgenin* (Kamm)	205-207 (205-207)	50	2	0	0	
36 *	Etiocholane-3(β)-ol-17-one, *3(β)-etiocholanolone* (Kamm)	151-153 (152-154)	50	3	0	0	
37 E*	Etiocholane-3(β)-ol-17-one acetate (Kamm)	157 (157-159)	50	3	0	0	

TABLE 1—*Concluded*

NO.	NAME	M.P.	DOSES	NO. OF ANIMALS	RESPONSE	MG./I.U.	REFERENCES
		°C.	mg.				
38 •	17-Ethyl-etiocholane-3(α), 20(α)-diol, *pregnanediol* (Cook)	237-238 (137-239)	50	3	0	0	in.: 5
39	17-Ethyl-etiocholane-3,20-dione, *pregnanedione** (Beall)	121 (120)	10 50	1 3	0 0	0	in.: 5, 22
40	17-Ethyl-etiocholane-3,20-dione-5,6(α)-diol-6-acetate, *pregnanedione-5,6(α)-diol mono-acetate*	218-219 (215-218)	7	1	0	0	
41	17-Ethyl-etiocholane-3,6,20-trione-5-ol *pregnane-3,6,20-trione-5-ol*	262-264 (262-268)	2	1	0	0	
42	17-Ethyl-Δ^{11}-etiocholene-3,20-dione, *Δ^{11}-pregnenedione* (Kamm)	190 (200-201)	50	3	0	0	
43	17^{a}-Methyl-Δ^{5}-chryso-pregnene-3(β),17^{a}-diol-17-one (Stavely)	180-182	5 10	1 1	0 0	0	
44	17^{a}-Epimer of above (Stavely)	275-278 (276-278)	5	2	0	0	
45	*Pseudo-sarsasapogenin* (Kamm)	164-170	50	3	0	0	

Symbols used: 0, +, ++, +++, ++++ = degree of activity according to McPhail's scale. † = directly injected into the uterine cavity; in. = inactive; sl. = slightly active; med. = of medium activity; high = highly active.

active compounds. It was not feasible to classify the literature more precisely because of the inadequacies mentioned in the introductory paragraph of this communication. It must be stated, furthermore, that due to the nondescriptive terminology employed by many workers we cannot state with certainty that the data are in every case recorded in connection with the compound actually used by these authors. In many instances, where the designations were inadequate, we merely mentioned the reference under the most common representative which could be designated by the term given in the original paper.

Perusal of the table shows that progesterone is by far the most active luteoid but, contrary to common belief, among the two next most active steroids ethinyltestosterone (also referred to as pregneninolone) is not significantly more potent than desoxycorticosterone acetate.

Concerning the correlations between the luteoid and other pharmacological actions of the steroids it may be said that in the sense of the classification given in a previous review (2) the luteoid effect is an independent main pharmacological action. This is indicated by the following facts: It is not subordinate to the testoid effect since progesterone itself is devoid of testoid activity (29)

while testosterone propionate, the most active testoid known, possesses only slight luteoid properties. It is not subordinate to the corticoid effect since ethinyl-testosterone is not corticoid (30) though highly luteoid. There is obviously no close correlation between the luteoid and the folliculoid activities since in high concentrations compounds exhibiting the latter effect actually inhibit the former action. Progesterone is a fairly potent spermatogenic compound as judged by its ability to prevent the destruction of the seminal epithelium caused by folliculoids, but since neither desoxycorticosterone acetate nor ethinyl-testosterone share this effect (31) no close correlations can exist between the luteoid and the spermatogenic actions. Although progesterone possesses high anesthetic properties, the latter are also evident in pregnanedione and pregnanediol (30) both of which are devoid of luteoid activity. Finally there is no close correlation between the renotropic and the luteoid activities since progesterone is considerably less renotropic than testosterone although the latter exhibits weaker luteoid properties. Thus it may be said that our observations support the view (2) that the luteoid effect is an independent action which can be separated from the other six main independent steroid hormone effects namely the folliculoid, testoid, corticoid, spermatogenic, anesthetic and renotropic potencies.

With regard to pharmaco-chemical correlations only a few generalizations can be made as yet. No compound possessing a hydrogen at C_5 in "*cis*" position to the C_{10} methyl group (as in etiocholane or pregnane) possesses any luteoid activity, but compounds with the C_5 hydrogen in "*trans*" position to the above methyl group as well as Δ^4 or Δ^5 unsaturated compounds which have no hydrogen at C_5, may exhibit luteoid activity.

No compound is known to exhibit luteoid potencies if it possesses a side-chain of more than 4 carbon atoms. However, the example of *21*-ethyl-progesterone indicates that a butyl side-chain is not incompatible with a high degree of this potency. Complete absence of a side-chain, on the other hand, is compatible with luteoid activity as shown by testosterone, androstenedione, etc.

Neither of the two ketone groups of the progesterone molecule is essential for its effect. Ethinyl-testosterone has no C_{20} ketone group and both pregnenolone and acetoxy-pregnenolone are devoid of the C_3 ketonic oxygen, yet all these compounds are luteoid.

The introduction of a C_{21} acetoxy group increases the corticoid but simultaneously decreases the luteoid actions of progestationally active steroids. This is true both of the Δ^4-*3*-one series (compare progesterone with desoxycorticosterone acetate) and of the Δ^5-*3*-(β)-ol series (compare pregnenolone with acetoxy pregnenolone) (30, 32–35).

A certain degree of oxygenation is essential for luteoid activity since the completely hydrogenated parent hydrocarbon androstane proved completely inactive while androstanedione, a saturated diketone, and androstanol, a saturated alcohol of the above compound, both exhibited definite luteoid properties. It is surprising that neither we nor previous investigators were able to elicit progestational changes with androsterone. In view of the activity of andros-

A SPLENIC CONTRACTING SUBSTANCE IN ORANGE SEEDS

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The interest in this project arose from the use by one of us (F. T. M.) of a crude extract of orange seeds in the treatment of malaria in Ecuador. One effect which was observed was contraction of the enlarged spleen of patients with this disease. The present paper concerns a description of the preparation of the extract, its partial purification, and its pharmacological action with particular reference to its effect on the spleen.

PREPARATION OF THE CRUDE EXTRACT. Two kilograms of whole dried orange seeds were placed in a cotton sack and extracted in 10 liters of boiling water for one hour. The extraction was repeated twice with 10 liters for one hour and then twice with 5 liters for 45 minutes, thus involving a total volume of 40 liters of water and a total extraction period of four and one-half hours. The combined aqueous extracts were evaporated over an open flame to a volume of approximately 8 liters, when the brown solution became somewhat syrupy in consistency. Evaporation was continued over a steam bath until the solution was semisolid. On cooling 600 grams of a soft brownish-black gummy product, containing approximately 40% water was obtained, this will henceforth be referred to as the *crude extract*. This material gradually forms a colloidal solution with the addition of water, solution being facilitated by heating. A certain amount of insoluble material may settle on standing or may be removed by centrifugation. The solution of the crude extract is bitter, has a pH of approximately 4.5, titratable acidity of 3 7 cc. $\frac{N}{10}$ NaOH per gram, and reduces Benedict's solution.

TOXICITY. The acute toxicity of the crude extract was determined in mice by administering by subcutaneous injection a solution containing 0.25 grams of the crude extract per cc. The results shown in table 1 indicate a relatively low toxicity. The $L.D._{50}$ is approximately 7 mgms. with a surely fatal dose of 10 mgms. and a safe dose of 5 mgms , per gram of body weight. The significance of these results lies in the fact that dosages of a definitely lower order were used on dogs to produce the effects to be described.

The respiration of all mice became slower and deeper within two minutes after injection and with the large doses there was definite dyspnea. The respiratory changes were accompanied by lessened activity and apparent depression and weakness Although the animals showed definite irritability or resentment on being touched they did not respond to noises or jarring of the table. A few of the mice which died showed an increase in activity just before death with convulsive movements of tail and legs. The mice (one in each of 3 groups) not included in the average time of death in the table died several hours later than the others. Eleven additional mice injected with crude extract (7.5–11.25 mgm. per gram of body weight) neutralized with sodium hydroxide showed the same manifestations and only one recovered.

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ACTION OF THE CRUDE EXTRACT. On intravenous injection of a total dose of 0.05–0.5 grams of the crude extract in solution, into 8–18 kgm. dogs anesthetized with sodium pentobarbital (35 mgm. per kgm. of body weight by intravenous injection) a contraction of the spleen occurred, as recorded from an oncometer. The contraction began within a few seconds after injection and was maintained for various periods, the duration of the response varying with the dosage and with the dog. Occasionally the spleen expanded to normal size within a few minutes and the response could be repeated several times in the same animal. Sometimes with larger or repeated doses the contraction persisted for hours. At the conclusion of an acute experiment of from two to five hours in duration, direct observation showed the spleen to be very firm and contracted to one half or less of the original size.

The character of the response was not constant but there was commonly observed an abrupt contraction followed by a dilatation lasting for from two to five minutes and then a gradual persistent contraction (table 2, Expt. 1). A second type of response, which was more commonly seen with smaller doses

TABLE 1

The toxicity, by subcutaneous injection, of the crude extract of orange seeds in mice

NO. OF MICE	DOSE (MGM./GM. BODY WEIGHT)	DIED	AV. TIME OF DEATH
			minutes
5	20.0	5	68 (4 mice)
5	10.0	5	181 (4 mice)
5	7.5	4	137 (3 mice)
5	6.25	1	50
10	5.0	0	

and with our partially purified extracts, was an abrupt splenic contraction with comparatively rapid and complete recovery. On the other hand some partially purified products showed only the gradual prolonged splenic contraction without the abrupt phase (see fig. 1).

Strong splenic contraction has also been observed after administration of one gram of the crude extract by stomach tube (table 2, Expt. 4). In this connection it is remarkable that the action began within a minute after administration. A reflex effect is suggested since it is difficult to conceive of absorption within this time.

Immediately after intravenous injection there was usually a fall in blood pressure which returned to normal or temporarily above normal, but if the injection was made slowly the splenic contraction might occur without any appreciable change in blood pressure (table 2, Expt. 3 (a)). The action on the spleen therefore appears to be independent of blood pressure changes. Variations in kidney volume, when they have been recorded, were also of a temporary character and seem more closely related to circulatory changes. A common observation was an increase in pulse pressure which became more marked on repeated injections and persisted throughout the experimental period.

The action on the spleen was as a rule only partially inhibited by atropine but very large doses (2–3 mgm. per kgm.) have eliminated it in some animals.

On intravenous injection there was a stimulation of respiration, with reference to both depth and rate, which may be followed by a period of apnea.

PURIFICATION. Since it was obvious that we were using a preparation containing large amounts of inert material and possibly more than one active principle we considered it advisable to attempt some purification before undertaking a detailed pharmacodynamic study. In this work the various fractions

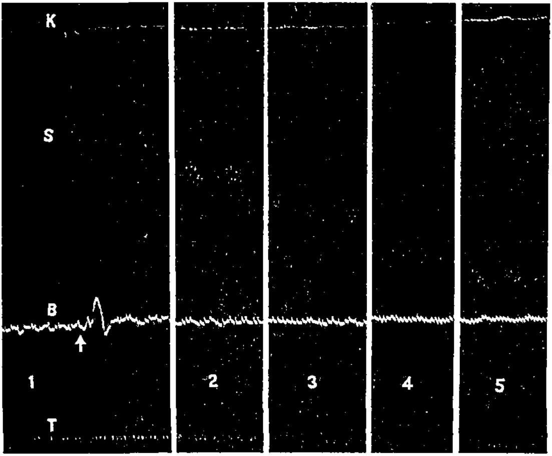

FIG 1. SLOW PROLONGED SPLENIC CONTRACTION AFTER INJECTION OF AN ALCOHOL SOLUBLE ETHER INSOLUBLE FRACTION

At the arrow the product of 0 25 gms of the crude extract was injected intravenously into a 9.5 kgm dog *K* kidney volume. *S:* spleen volume. *B* blood pressure. *T:* time—5 seconds. Time after injection of record No 2—11 min No 3—21 min No. 4—33 min No. 5—37 min.

obtained were assayed by their activity in producing splenic contraction. A total of 40 dogs were used for the purpose; in table 2 are summarized the results of 9 representative experiments

Considerable difficulty was experienced in purification on the basis of differential solubility due to the fact that the active principle and most of the inert material were much more soluble in water than in other solvents. The active substance is comparatively insoluble in ether, dry acetone, petroleum ether, chloroform, carbon tetrachloride and methylene chloride, and these solvents also failed to remove an appreciable amount of the inert material. An initial

step of some value was the filtration of the aqueous solution through bone charcoal. Water-clear solutions which were active in producing splenic con-

TABLE 2

The effect of orange seed extract on spleen volume and blood pressure

EXP. NO.	DOG WT.	SAMPLE OF EXTRACT*	SPLENIC CONTRACTION		BLOOD PRESSURE CHANGES			
					Fall		Rise	
			Degree	Duration	Maxim.	Duration	Maxim.	Duration
	kgm.			*min.*	*mm. Hg*	*minutes*	*mm. Hg*	*minutes*
1	9.0	Crude ext., 0.5 gram	(volume increase) marked; gradual	4 60+	50	1		
22	9.5	Crude ext., 0.14 gram	moderate; abrupt moderate; gradual	2 20+			10	3
3a	18	Crude ext., 0.06 gram	moderate	5	none		none	
3b		Crude ext., 0.06 gram	marked, abrupt		10	1		
4	13	Crude ext., 1.0 gram orally	marked; gradual for 1 hour	no recovery	none		none	
5	10	Charcoal filtrate, 0.55 gram	marked, abrupt	3	25	1	10	8
6	8	$CHCl_3$ ext. of $Ca(OH)_2$ filtrate, 0.5 gram	slight	1.5	5	0.25	5	0.25
		$CHCl_3$ insol. fr. of above, 0.2 gram	marked; abrupt	22	25	1.5		
7	16	Crude ext., 0.25 gram	marked	6	25	0.5		
		Alcohol sol. fr., 0.25 gram	marked	3	40	1		
		Alcohol insol. fr., 0.5 gram	slight	2	none		none	
		Resin (?) fr., 0.25 gram	no effect		none		none	
8	10	Ether ext., 0.4 gram	moderate, recovery incomplete				45	4
		Ether insol. fr., 0.1 gram	marked, abrupt (volume increase) moderate	1 2 10	50	1		
9	9	Alcohol sol., ether insol. fr., 0.25 gram	marked; gradual for 37 min. no recovery	60+			25	0.3

* The dosage cited for the partially purified extracts refers in each case to the amount of crude extract from which it was obtained.

traction have been obtained in this way (Table 2, Expt. 5) but the process was extremely time consuming, due to the necessity of repeated and slow filtrations. Furthermore certain samples of charcoal seemed to be definitely less effective than others. An alternate process was the saturation of a 2% aqueous solution

of the crude extract with calcium hydroxide, which resulted in the formation of a relatively inert gel which could be removed by filtration.

Considerable amounts of inert material could be precipitated by alcohol, the amount of precipitate increasing with the alcoholic concentration. The active material remained in solution with alcoholic concentrations up to 60 or 70%. Above this, increasing amounts of activity were found in the precipitate although some remained in solution in comparatively large volumes of 95% alcohol (attained by the addition of twenty volumes of absolute alcohol to aqueous solutions). On evaporation of the alcoholic solution to dryness the portion containing all activity could be dissolved in water leaving an insoluble inert fraction, presumably of a resinous nature (table 2, Expt. 7). By this procedure, however, a considerable amount of the activity was lost, and this could not be accounted for entirely by that remaining in the precipitate, especially when the alcohol was removed by evaporation on a boiling water bath rather than by the use of an air current at room temperature.

A method which gave some promise of success was the initial treatment of the crude extract with calcium hydroxide as described above, reduction of the volume of the filtrate to approximately one-tenth on a boiling water bath, the addition of absolute alcohol to give an alcoholic concentration of ninety % by volume, and then the removal of the precipitate by centrifugation. When the decanted fluid was treated with two volumes of ether a precipitate was obtained which could also be removed by centrifugation and which contained considerable activity (see fig. 1 and table 2, Expt. 9).

Fractionation with basic lead acetate was also attempted. Although more activity was obtained in the precipitate than in the filtrate the procedure did not appear to be very efficient.

On the supposition that the active principle might have alkaloidal properties, attempts were made to extract it from aqueous solutions made alkaline with ammonia or sodium carbonate. Ether was used more successfully than chloroform but extraction, even when prolonged (from 5 to 10 hours extraction in a Watkin's extractor), was incomplete and was not facilitated by saturation with sodium chloride. The active substance could readily be removed from the ether by shaking it in a separatory funnel with acidified water. Most of the extracts obtained in this way produced, in addition to splenic contraction, an abrupt rise in blood pressure. The residue remaining from ether extraction was even more potent in producing contraction of the spleen but caused a marked fall in blood pressure (table 2, Expt. 8). In some cases a dose obtained from 0.1 gram of crude extract produced a fall in pressure to 10–15 mm. Hg, recovery occurring in from 10 to 15 minutes, that is, much more slowly than in the experiment cited in table 2. This suggests the presence of more than one principle having an effect on blood pressure since a pressor principle was extracted by ether while the residue, containing most of the inert material of the crude extract, produced the opposite effect. The moderate and somewhat variable effect on blood pressure of the crude extract itself could be explained by a combination of the two effects.

With the possibility in mind that the extract contained certain organic acids, some of which had physical properties similar to those of the active principle, the following were found to be inactive in a 10 kilogram dog on intravenous injection: ascorbic acid 30 mgm. and 50 mgm., malic acid 20 mgm., citric acid 20 mgm. and succinic acid 20 mgm.

Discussion. Although it has been definitely shown that the extract produces a strong contraction of the spleen the experiments on purification are considered as attempts rather than accomplishments. Most of the procedures were relatively inefficient in that both fractions obtained in the various steps usually contained activity. However, definite progress is illustrated by the fact that the calcium hydroxide-alcohol-ether treatment described above yielded a product ten times as active as the crude extract per unit weight. Due to the difficulties in assay, such comparisons are crude approximations. The spleens of various animals showed considerable differences in sensitivity and if comparisons are made on the same animal definitely effective doses gradually made the spleen less responsive until it apparently was incapable of further contraction.

SUMMARY

A crude extract of orange seeds contains a water soluble substance of comparatively low toxicity which produces strong splenic contraction. Although an active principle has not been isolated definite progress has been made in its purification.

ACTIONS OF A SERIES OF DIPHENYL-ETHYLAMINES

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In spite of the innumerable studies of the sympathomimetic amines reported in the last forty years, the series of *1,2* diphenyl-ethylamine substituents does not appear to have been systematically investigated. These compounds, which are the secondary amines obtained by the addition of various aliphatic groups to the primary amine, have been recently synthesized, and supplied us for study, by Drs. C. W. Sondern and C. J. Wesley Wiegand as hydrochloride salts. The diphenyl compounds which have been studied previously have been mainly depressor to the circulation. The most promising lines of investigation, therefore, seemed to be the bronchodilator effects, if any, and a possible central nervous system stimulation of the benzedrine type. This report shows that, while these products possess some activity, their potency does not indicate a field of clinical usefulness along the lines investigated. Nevertheless, the results obtained by us are of scientific interest, and therefore worthy of a condensed report.

COMPOUNDS STUDIED. The compounds studied were *1,2* diphenyl-ethylamine and its methyl, ethyl, normal propyl, isopropyl and isobutyl derivatives, together with two compounds with *p*-methoxy groups added to make anisyl rings. The structural formulae and the toxicities, as determined elsewhere (1), are given in table 1. The hydrochlorides are white crystalline salts readily soluble in water, and stable on standing in aqueous solution.

BRONCHI. Guinea pigs lungs were perfused by a method previously described (2). Briefly, warm perfusion fluid was passed into the trachea of excised lungs, after scratches had been made on the pleural surfaces of each lobe through which the fluid escaped after traversing the bronchial tree. The fluid was quantitatively collected each minute, and the changing flow recorded as a measure of bronchoconstriction or bronchodilatation. With this technic, doses of epinephrine of 0.2 cc. of 1:10,000 or more injected into the perfusion system at the lungs caused prompt dilatation lasting 10 minutes, while histamine, 0.2 to 0.5 cc. of 1:10,000, caused vigorous constriction. When the lungs were sensitized by previous injection of the guinea pigs with horse serum, injection of the antigen into the perfusion fluid caused prompt bronchospasm in the usual manner.

All the diphenyl compounds listed in table 1 were injected into the perfusion system hear the lungs in doses of 0.2 to 2.0 cc. of either 0.5 or 1% strength. Only the ethyl-diphenyl derivative produced any dilatation, and this was variable. The others were moderately effective broncho-constrictors, acting apparently by causing a spasm of the smooth muscle directly, since the contraction was not prevented by previous atropinization. However, the spasm could be relieved by epinephrine, or aminophylline. When the new amines were injected into bronchi

[1] Supported, in part, by grants from the George A. Breon Co., Inc.

already partially constricted by antigen, further constriction occurred. Tachyphylaxis was rather marked, and this made it difficult to get more than one or two satisfacrory responses from each lung.

In 2 dogs, Jackson's negative pressure method was used (6), administering erythrina for curarization instead of pithing. The diphenylethylamine and ethyl substitution product in doses of 5 to 15 mgm. per kg. intravenously regularly produced a fall of blood pressure which sometimes was followed by a rise of 20 to 30 mm. Hg. The bronchi were invariably constricted, although not to a marked degree. A typical record is shown in figure 1.

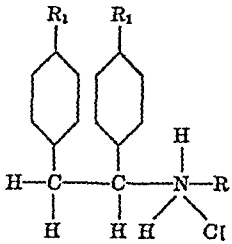

TABLE 1

List of compounds studied, and their reported toxicity (1)

(The lethal doses were computed according to Behm's method (Burn: Biological Standardization, 1937, Oxford Press) and are reliable to within ±5 mgm).

COMPOUND NUMBER	SUBSTITUENT IN DIPHENYLETHYLAMINE		LETHAL DOSE INTRAVENOUSLY IN WHITE RATS		
	R	*R₁*	LD_1	LD_{50}	LD_{99}
			mgm. per kg.	*mgm. per kg.*	*mgm. per kg.*
I	H	H	30	45	60
II	CH_3	H	40	60	80
III	C_2H_5	H	40	55	70
IV	C_3H_7(n)	H	30	45	60
V	C_3H_7(iso)	H	30	45	60
VI	C_4H_9(iso)	H	45	65	75
VII	H	OCH_3	40	50	70
VIII	C_2H_5	OCH_3	25	40	50

In another dog in which blood pressure and spontaneous respiration were recorded, diphenylethylamine and its *N*-propyl derivative in doses of 1 to 15 mgm. per kg. intravenously, caused a marked fall of blood pressure with accelerated heart and decreased respiration. These effects were not prevented by full atropinization, and probably were the result of direct cardiac depression. In view of the failure to demonstrate, in intact dogs and in isolated guinea pig lungs, responses which might indicate potential clinical value for the compounds, the observations on the respiratory and circulatory systems were not extended.

CENTRAL NERVOUS SYSTEM. The effects on the central nervous system were studied in two ways:—first, by measuring cortical excitability of rabbits, and

second, by recording spontaneous activity in rats following administration of a range of doses of the compounds.

Cortical excitability. The excitability of the cerebral cortex of normal rabbits was determined by measuring the density of current required to cause convulsive seizures. For this purpose a metal bit was put in the mouth as one electrode and a metal plate on top of the head as the second electrode. Contact on the vertex of the head was made more efficient by clipping the hair and putting a small rubber sponge wet with salt solution under the electrode. These electrodes were anchored firmly in place by elastic bands which went around the ears and prevented the electrodes from slipping when the animals had convulsions. To the electrodes were clipped flexible leads coming from a specially designed "high

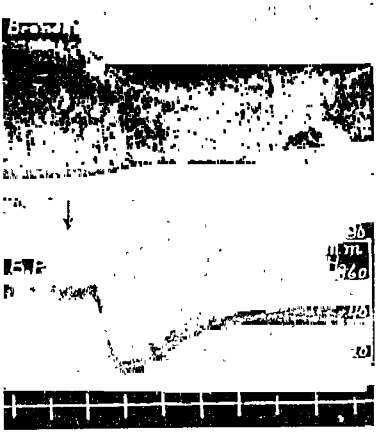

FIG. 1. EFFECT OF DIPHENYLETHYLAMINE ON BRONCHIAL TONUS AND BLOOD PRESSURE
Dog, 8.5 kg. Pentobarbital anesthesia, and erythrina for curarization. Jackson's technic. 15 mgm. per kg of diphenylethylamine HCl caused a fall of blood pressure and bronchoconstriction. Time = 20 sec

resistance" stimulator. The details of this apparatus will be described in another report. In essence it consisted of a fixed step-up transformer, fed a voltage from a variable transformer connected to a 110 volt 60 cycle current source On the output side were fixed resistors of 10,000 ohms, so that the 50- to 100-ohm fluctuations in skin resistance at the electrodes caused by the rabbit's movements did not demonstrably change the current flow. Two milliammeters with 6 inch scales, having a range of 0 to 25 and 10 to 100 milliamperes (ma), respectively were used to measure the current.

To make a determination, the electrodes were put on and connected to the stimulator. Then current was passed for exactly 15 seconds at an amperage of about 5 ma. below the expected threshold This current was readily adjusted to the desired level by regulating the

voltage output of the variable transformer. After exactly 5 minutes' rest, a current about 1 or 2 ma. higher was tried, and this procedure was repeated until the threshold was reached. As the current was increased in strength motor excitation from current flow through the motor cortex was seen; this was synchronous with the 60 cycle current and started and stopped instantly with the current. The true epileptiform convulsions, used as the end point, were focal in origin and spread over the body, Characteristically, the convulsions did not begin until from ½ to 1 minute after the current-flow was stopped. They lasted 2 or 3 minutes, and left the cortex depressed so that no further determinations could be made that day. If the true threshold had been exceeded by using too great a current, the epileptiform convulsions were apt to begin before the stimulus was stopped, and close observation was required to recognize them in the presence of the direct motor excitation. However, the latter stopped abruptly with the current, whereas the epileptoid activity persisted and had a different pattern, so that differentiation was readily made after some experience.

From 5 to 7 rabbits were used simultaneously in a group, each group being used about once a week. At the beginning, the thresholds were determined repeatedly without medication until consistent thresholds for each rabbit were obtained. The mean control values under these conditions were about 20 ma. with a standard error of the mean for each rabbit averaging about 0.5 ma. Thus the control levels were quite reproducible and stable in each animal. When the control level of excitability had been ascertained, the drug was administered and the threshold re-determined at a time estimated to coincide with the probable peak of effect of the drug. The difference between this threshold and the average of the controls for each rabbit was taken as the effect of the drug. These differences were averaged for the group and the standard error of this mean calculated to determine whether the mean difference was significant. For 5 rabbits, a 5% probability required a critical ratio of 2.78, for 6 rabbits, 2.57, and for 7, 2.45 (5). In table 2, these mean differences and their standard errors are summarized, from which the critical ratios and the probable significance of the differences can be readily estimated. Control determinations without medication were always alternated with those under medication, so that any persistence of drug action would be recognized and a longer recovery period allowed. Doses were started at 0.5 or 1 mgm. per Kg. subcutaneously and doubled successively until symptomatic changes or deaths indicated the limit of tolerance had been reached.

In table 2 are summarized the effects of ethyl alcohol, which is commonly recognized as a cortical depressant, for comparison with the new drugs studied. One cc. of alcohol per kg. gastrically produced a threshold degree of depression, which increased progressively with 2 and 4 cc. With the larger dose, the rabbits were ataxic and depressed. Picrotoxin was included as a typical excitant; this lowered the threshold 5 ma. in dosage of 1 mgm. per kg. subcutaneously.

In contrast to alcohol and picrotoxin, diphenylethylamine, and its methyl, ethyl, normal propyl and methoxy derivatives caused no consistent changes of sufficient magnitude to be related to the doses administered. The isopropyl derivative raised the threshold 3.7 ma. with a dose of 8 mgm. per kg. subcutaneously but at this level there was marked spontaneous motor ataxia, indicating a generalized depression. The isobutyl compound lowered the threshold, but required 16 mgm. per kg. to produce a significant degree of change. The ethylmethoxy derivative in a 1 mgm. dosage depressed the cortex, but doses of 8 and 16 mgm. made the cortex more excitable. But, because of the inconsistencies with the remaining doses, the conclusion was drawn that this compound had no significant effect.

In view of the clear-cut alterations in cortical excitability readily demonstrated

with known stimulant and depressant drugs, in contrast to the inconstant and minor changes with dephenyl-ethyl amines tested, these compounds have no important effect on cortical function under these conditions.

Spontaneous activity. To measure the effect of these compounds on spontaneous activity, white rats were suspended in the "jiggle cages" described previously (3) and their movements recorded after injection of from 1 to 128 mgms. per kg. subcutaneously. Ten rats were used for each dose, the average activity being calculated for each half-hour and corrected to give the net increase by subtracting

TABLE 2

Effects of alcohol, picrotoxin and diphenylethylamine compounds on the excitability of the cerebral cortex of rabbits to electrical stimulation

Five to seven animals were used for each dose

COMPOUND NUMBER	MEAN CHANGE IN THRESHOLD IN MILLIAMPERES WITH STANDARD ERROR OF THE MEAN CHANGE ACCORDING TO DOSE IN MGM. PER KILOGRAM SUBCUTANEOUSLY*									
	0.125 mgm	0.25 mgm	0.5 mgm.	1 mgm	2 mgm.	4 mgm.	8 mgm	16 mgm	32 mgm.	64 mgm
Ethyl alcohol			+1.18 ±0.91	+1.62 ±0.71	+4.78 ±1.19	+16.28 ±1.10				
Picrotoxin	−1.18 ±0.81	−1.76 ±0.38	−3.90 ±0.86	−5.04 ±1.43	Convulsant					
I				+0.98 ±0.69	+1.28 ±0.74	+0.28 ±0.80	+0.84 ±0.43	+1.98 ±0.91		
II				+0.52 ±0.28	+2.41 ±0.89	+1.48 ±0.85	−0.94 ±0.82	−0.1 ±0.84		
III				−0.24 ±1.23	+1.36 ±0.40	+3.60 ±1.17	+2.06 ±1.26			
IV				+0.71 ±0.61	+0.53 ±1.06	+1.90 ±0.36	+2.66 ±1.28			
V			−0.18 ±0.69	+2.01 ±0.43	+1.18 ±0.38	+3.07 ±0.65	+3.70 ±1.31			
VI				0 ±0.73	−1.13 ±1.41	−0.31 ±0.83	−0.5 ±1.43	−4.34 ±0.86		
VII				+0.60 ±0.26	+0.75 ±0.56	+1.16 ±0.55	+2.10 ±1.20	−0.34 ±2.80	+1.06 ±0.53	+1.95 ±1.89
VIII			−0.44 ±0.49	+2.20 ±0.52	−0.10 ±1.64	+1.0 ±0.99	−4.38 ±1.26	−5.36 ±0.74	+0.38 ±0.91	

* Ethyl alcohol in cc. per kilogram intragastrically. + indicates the threshold was raised and − that it was lowered the number of ma. recorded.

the spontaneous activity in parallel control runs on the same rats. The doses were started at 1 mgm. per kg. and doubled at each successive weekly experiment until a dose of 128 mgm. had been administered. This total dosage killed none of the rats, showing that considerably higher doses are tolerated subcutaneously than the fatal intravenous doses reported in table 1.

The two methoxy-compounds were practically inactive, since compound VII caused no stimulation in any dose and its ethyl derivative (VIII) barely exceeded a threshold effect at the 128 mgm. dose level. Also inactive was the isobutyl derivative of diphenyl-ethylamine (VI).

All the other compounds caused definite excitation, resembling qualitatively that of benzedrine, caffeine, and similar compounds whose actions have been previously described (3). The effects of doses of 128 mgm. are charted in figure 2. It is seen that, in comparison to benzedrine, these compounds have a low order of

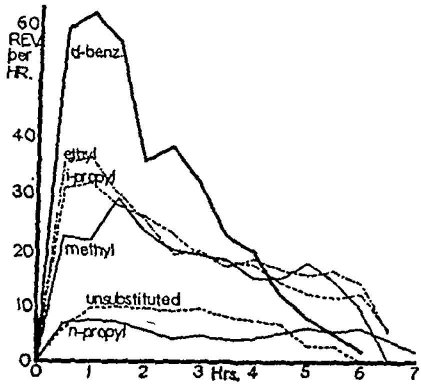

FIG. 2. COMPARATIVE INCREASE IN SPONTANEOUS ACTIVITY PRODUCED IN WHITE RATS BY DIPHENYLETHYLAMINE HYDROCHLORIDE AND ITS METHYL, ETHYL, NORMAL PROPYL AND ISOPROPYL ADDITION PRODUCTS, AND *d*-BENZEDRINE SULFATE

Each curve shows the average net increase in activity of 10 rats injected with 128 mgm. per kilogram of the diphenyl-compound or 4 mgm. per kilogram of *d*-benzedrine sulfate, as recorded in revolutions of the work adder per hour.

TABLE 3

Threshold doses and maximum effects of diphenylethylamine compounds compared with d-benzedrine on spontaneous activity of white rats

The drugs were given subcutaneously

COMPOUND NUMBER	THRESHOLD DOSE	MAXIMUM PEAK EFFECT	
		Dose	Revolutions per hour
	mgm. per kg.	*mgm. per kg.*	
I	4	128	10
II	16	128	29
III	8	128	36
IV	16	128	7.6
V	4	128	32
VI	No stimulation		
VII	No stimulation		
VIII	128	128	6.2
d-benzedrine sulfate	0.25	2	62

activity, since the stimulation of 128 mgm. is not as great as that of 4 mgm. of *d*-benzedrine.[2] The same lack of potency is shown in the threshold doses, summarized in table 3. The duration of action of all compounds was between 6 and 7 hours, with the more effective doses.

[2] The data on *d*-benzedrine are taken from a paper by Novelli and Tainter (in press).

Accordingly, these derivatives possess a central stimulant potency somewhat less than that of ephedrine and still less than that of benzedrine or its methyl derivative, "pervitin." It is interesting to note that the secondary amine formed by methylation or ethylation of the amino group increased the potency of the diphenyl compounds in a manner similar to that for phenyl-isopropylamine (4). Even the isopropyl derivative is stronger than the primary amine, but not so the isobutyl compound. However, in spite of the definite potency of these agents as central nervous system stimulants, the high dosage required for such action is unlikely to offer an adequate margin of safety for clinical use. This is further indicated by the undesirable bronchoconstrictor and circulatory depressor effects of considerably smaller doses.

SUMMARY AND CONCLUSIONS

1. The important systemic actions are described of a series of diarylethylamines, consisting of secondary amines derived by substitution on *1,2* diphenylethylamine, including two products with p-methoxy groups or anisyl rings.

2. The intravenous LD_{50} of these compounds for white rats is in the range of 50 mgm. per kilogram of body weight.

3. Doses in the lower range cause weak and irregular pressor action, and definitely effective doses cause prompt decreases of blood pressure with acceleration of the heart, which is unchanged by atropinization. Tachyphylaxis is very pronounced.

4. Bronchi *in situ* in dogs and in isolated guinea pig lungs are weakly constricted by these agents, and no antispasmodic action occurs in anaphylactic shock.

5. The threshold of excitability of the cerebral cortex is not consistently affected by these compounds, except by excessive doses which are unpromising for therapeutic use.

6. According to changes in spontaneous general activity of rats in "jiggle cages," diphenyl-ethylamine causes a slight stimulation after doses of 128 mgm. per kilogram, while equal doses of its methyl, ethyl and isopropyl derivatives produce about 3 times as great an effect. By contrast, 4 mgm. of *d*-benzedrine sulfate causes approximately twice the stimulation, which lasts almost as long.

Therefore, the general stimulant action of the diaryl ethylamines investigated is not great enough to be of clinical usefulness.

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THE STIMULANT POWER OF SECONDARY AND TERTIARY PHENYL-ISOPROPYL-AMINES[1]

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Phenyl-isopropyl-amine (also known as benzedrine, amphetamine, and isomyn) is known to have a relatively low order of activity for the circulation, but a most pronounced stimulant action for the brain. In view of the widespread clinical use of this compound, it would be desirable to know whether modifications in its structure might result in compounds of increased potency. One of us (T.) and associates have previously reported on the central effects of over seventy sympathomimetic amines, including compounds exemplifying nearly every variation in the basic chemical structure (1, 2). However, secondary and tertiary amines were not adequately represented in those investigations. A study of these amines seemed particularly desirable in view of the special interest in the methyl derivative now being widely used as a clinical stimulant in Europe, under the names of pervitin, methyl benzedrine, desoxyephedrine, or methyl isomyn. Such a study was undertaken by us and the results obtained are presented in this report.

One of us (N.) synthesized the methyl, ethyl, butyl, amyl, dimethyl and diethyl derivatives of phenyl-isopropyl-amine and also the piperidyl substituent for the amine group. These compounds were used as the racemic hydrochlorides (melting points and analytical data in table 1). In addition, we were supplied, through the courtesy of the Abbott Laboratories, the dextro and levo isomers of methyl benzedrine and an additional sample of the racemic mixture of the same compound. The levo-product was an acid tartrate salt, so that in solutions for injection an amount was used to make the solution equivalent to the hydrochloride. Dextro-benzedrine sulfate, supplied by Smith, Kline and French, was included in this study to afford a basis of comparison with results previously published (1, 2).

CENTRAL NERVOUS STIMULATION. The ability of these compounds to stimulate the central nervous system was compared with that of *d*-benzedrine, according to the method previously described by one of us (1, 3). Briefly, an unanesthetized rat is confined in a small cage hanging from sensitive springs. As the rat moves restlessly about the cage, the movements of the springs are transmitted to a work adder, which closes an electrical circuit with each revolution, thereby giving a kymograph record of the integrated activity per unit of

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time. Twenty such cages are suspended in a lighted glass chamber kept at a constant temperature of 27°C., so that variations in spontaneous activity, due to room temperature and light, will not affect the results.

The normal activity of all the animals was recorded on alternate days, or one-half the animals each day, until a highly reliable estimate of the spontaneous activity was available. This activity was calculated separately for each half hour period and the average values subtracted from those after the action of the drug to give the net increase over the normal level. Ten rats were used for each dose on any one day, and the average increases for each half hour calculated. The rats were used repeatedly at intervals of between one and two weeks. All the doses were calculated in terms of the body weight and are here reported in mgms. per kilogram. A dose of 1 mgm. was given subcutaneously as the beginning dose of each compound. If stimulation resulted at this level, the dose was

TABLE 1

Physical and chemical characteristics of products studied

FORMULA	MELTING POINT	OPTICAL ROTATION	CHLORIDE	
			Calculated	Found
	°C.		per cent	per cent
d-phenyl-*1*-amino-*2*-propane sulfate	306–307			
dl-phenyl-*1*-methylamino-*2*-propane HCl	132–133		19.13	18.95
d-phenyl-*1*-methylamino-*2*-propane HCl	171–173	+14°		
l-phenyl-*1*-methylamino-*2*-propane acid tartrate	159–160	−14°		
dl-phenyl-*1*-ethylamino-*2*-propane HCl	145–146		17.79	17.75
dl-phenyl-*1*-*n*-butylamino-*2*-propane HCl	168–169		15.68	15.70
dl-phenyl-*1*-*n*-amylamino-*2*-propane HCl	186–187		14.69	14.55
dl-phenyl-*1*-dimethylamino-*2*-propane HCl	156–158		17.79	17.75
dl-phenyl-*1*-diethylamino-*2*-propane HCl	160–161		15.63	15.40
dl-phenyl-*1*-piperidyl-*2*-propane HCl	206–208		14.72	14.80

successively halved until no stimulation was observed. Higher doses than 1 mgm. were then administered by successively doubling the dose until either death occurred or it was impracticable to increase the dosage further. The highest dose used was 128 mgm. and, when no stimulation occurred, no further administration of the drug was attempted.

Dextro-benzedrine was included as a control and for comparison with the results previously reported. This was necessary because, since the publication of the previous paper (1), the springs on these cages had been altered in sensitivity, and the absolute magnitude of the responses in that paper could not be compared directly with those in this paper.

The threshold dose required for definite stimulation was the smallest amount which produced an average total increase in activity in the first 4 hours greater than 3 standard deviations more than the average activity observed in the control series. However, since the dosage intervals were fairly wide, the threshold dose could not be considered as established more than within the

range of the reported values. The dose which produced the average maximum peak increase in activity was recorded as the maximum average rate of revolutions of the work adder per hour observed over any half hour period of recording. The maximum total effect was reported as the greatest average increase in total activity produced by a given dose, recorded from the time of injection of the drug until the activity returned to normal. Again this value was smaller with *d*-benzedrine in this study than it was previously, thus confirming the changed sensitivity of the spring suspension mechanism.

TABLE 2

The average central stimulant activity in 10 unnarcotised white rats of benzedrine derivatives injected subcutaneously

(All doses are expressed in mgm. per kilogram of body weight of the hydrochloride salt. In the fatal dose column are shown the lowest lethal doses and the proportion of animals killed.)

FORMULA	NAME	RANGE OF DOSES TESTED	AVERAGE THRESHOLD STIMULANT DOSE	AVERAGE MAXIMUM PEAK EFFECT		AVERAGE MAXIMUM TOTAL EFFECT		AVERAGE DURATION	PROPORTION KILLED AND DOSE
				Dose	Revolutions per hour	Dose	Total revolutions		
								hours	
d-phenyl-*1*-amino-*2*-propane	*d*-benzedrine	0.125–16	0.25	2	62	16	182	6.5	16, 1/10
dl-phenyl-*1*-methylamino-*2*-propane	*dl*-methyl benzedrine	0.125–8	0.25	2	57	4	135	6.5	8, 1/10
d-phenyl-*1*-methylamino-*2*-propane	*d*-methyl benzedrine	0.0625–16	0.125	0.5	54	0.5	133	7.5	16, 3/10
l-phenyl-*1*-methylamino-*2*-propane	*l*-methyl benzedrine	1–128	2	128	41	128	370	21	128, 0/10
dl-phenyl-*1*-ethylamino-*2*-propane	*dl*-ethyl benzedrine	1–64	4	16	52	32	127	6	64, 0/10
dl-phenyl-*1*-*n*-butylamino-*2*-propane	*dl*-butyl benzedrine	1–128	*						128, 0/10
dl-phenyl-*1*-*n*-amylamino-*2*-propane	*dl*-amyl benzedrine	1–128	128†	128	6	128	5	2	128, 0/10
dl-phenyl-*1*-dimethylamino-*2*-propane	*dl*-dimethyl benzedrine	1–128	8	64	34	64	114	7	128, 0/10
dl-phenyl-*1*-diethylamino-*2*-propane	*dl*-diethyl benzedrine	1–32	4	4	43	4	>125	>6	32, 0/10
dl-phenyl-*1*-piperidyl-*2*-propane		1–64	4	32	22	32	100	7	64, 1/10

* No stimulation.
† Convulsions.

In table 2 are summarized the results with the 10 compounds tested. The table shows that the peak effect of *d*-benzedrine in the present series was 62 revolutions per hour after a dose of 2 mgm. *D*-methyl benzedrine had a lower threshold dose than did *d*-benzedrine, and its peak effect was almost as great, but was produced by only one-quarter the dose required for the latter. The greatest total increase produced by *d*-benzedrine required 16 mgm., whereas almost as much total stimulation resulted from 0.5 mgm. of *d*-methyl benzedrine. Doses of *d*-methyl benzedrine greater than 0.5 mgm. not only failed to produce greater peak intensity of stimulation, but also showed a lesser total increase in activity,

indicating that there were depressant secondary effects which prevented the full development of the expected central stimulation. The racemic compound had about the type and degree of activity to be expected from its dextro-isomer content.

With doses of 1, 2 and 4 mgm. of *l*-methyl benzedrine, only small increases in activity occurred, amounting to peak effects of less than 11 revolutions per hour, while with 8 mgm. the peak effect was 30 revolutions per hour. But with higher doses the curve of response assumed a different form from that with any of the other amines previously discussed. That is, the peak effect, instead of occurring within the first 2 hours after the injection, was delayed progressively as the dose was increased until finally, at the level of 128 mgm., a peak of 41 revolutions per hour was reached between the eighth and ninth hours. A total of 21 hours was required for the activity to return to normal. It may be significant that, even with this large dose of 128 mgm. no deaths occurred, whereas the *d*-isomers of both benzedrine and methyl benzedrine produced fatal effects in concentrations only a fraction of the tolerated dose of the levo derivative. It would seem that, if a prolonged action of this general type were desired, it would be worth while studying in greater detail the applicability of *l*-methyl benzedrine for such a purpose.

Ethyl benzedrine was definitely weaker than methyl benzedrine, requiring higher doses and producing responses which did not persist as long. With the normal butyl and normal amyl derivatives, the typical stimulant activity was lacking. With the highest dose of the *n*-amyl compound tested, generalized convulsions were produced in 4 of the 10 rats, and these in no way resembled the type of activity produced by the other compounds. Dimethyl benzedrine was not as effective a stimulant as the secondary product, since both the degree of stimulation produced and the dosages required were many times larger than those of methyl benzedrine. With diethyl benzedrine, stimulation developed with 4 mgm. or more, but the effects came on so slowly that the activity was still increasing at the end of 6 hours, when the observations were discontinued. The data in table 2, therefore, do not reflect the full activity of diethyl benzedrine, and it is probable that both the peak effect and the total activity are greater than those indicated. The slow development of the responses to this product was not fully appreciated until the supply of the drug had been exhausted. Since the opportunity to extend the observations on this compound will not soon be available, the results are reported for what they are worth.

In the last product of the series, the amino group was replaced by the piperidine ring. This compound had moderate activity but neither its peak effect, total or duration of activity indicated that this particular modification of the molecule introduced qualities which were an improvement over those in the amino compounds.

It is concluded that *d*-methyl benzedrine, the compound called "pervitin," in Germany, and "methyl isomyn," in Great Britain, probably is more potent than the non-methylated compound, as indicated by the threshold stimulant dose. However, its action is not as intense as that of *d*-benzedrine, but the stimulation

is more sustained than that of the parent substance. For these reasons, therefore, the methyl derivative might be expected to give a better over-all increase in activity, particularly with doses and degrees of action that might be of interest clinically.

CIRCULATION AND RESPIRATION. Twelve experiments were performed on rabbits, cats and dogs under anesthesia to determine the respiratory and circulatory effects of these amines. More extensive observations were not made because it was found that the lack of activity on these functions did not warrant an extended study.

Methyl benzedrine produced small or negligible increases in blood pressure with doses of 1 and 2 mgm. intravenously. When the attempt was made to secure greater pressor effects by the injection of larger doses, only depressor responses were obtained. The weak and inconstant circulatory action indicated that the pressor dosage ratio to epinephrine would be somewhere between 1,000 and 2,000 (fig. 1). The first dose of methyl benzedrine was likely to give a

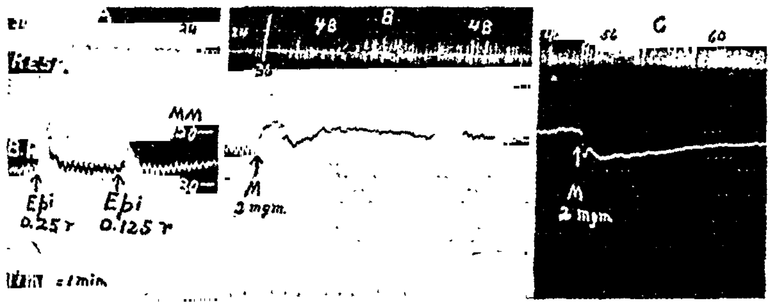

FIG. 1. BLOOD PRESSURE AND RESPIRATORY RESPONSES TO EPINEPHRINE AND PHENYLMETHYLAMINOPROPANE

Cat, 2.4 k., chlorbutanol anesthesia and atropinized. In *A* and *B* the responses to 0.125 and 0.25 micrograms of epinephrine and 2 mgm. of phenylmethylaminopropane are compared. In *C* the second dose of the latter compound, administered after a rest period of 2½ hours, gives a fall of blood pressure, demonstrating marked and persistent tachyphylaxis to even a minimal pressor dose.

rather persistent state of tachyphylaxis. This is illustrated in figure 1, in which a second dose of methyl benzedrine, administered 2½ hours after the first dose, is shown to produce only a depressor response. There had been no intervening doses of any drug except an occasional small test dose of epinephrine. The ethyl, butyl and amyl compounds showed no consistent pressor effects, and usually caused moderate depressions, if anything. In contrast, the dimethyl compound produced about as much increase in blood pressure as did the methyl derivative. The diethyl and piperidyl derivatives were practically inactive in any dose tested, except for depression in the higher dosage ranges.

The effect on respiration of these compounds was not very marked, with the possible exception of methyl benzedrine. This product usually produced an initial period of hyperventilation, in which both rate and depth of breathing were greatly increased. After the initial stimulation, there was usually a prolonged period of increased depth and rate of breathing, definitely less than the

initial stimulation, but persisting for some hours. Since this was associated with increased muscular activity, the respiratory stimulation may well have been part of the general excitation, recorded in the "jitter cages" on the rats.

In figure 1 it can be seen that the respiration, which was regular and at a rate of 24 per minute before the injection of methyl benzedrine, was doubled in frequency, and highly irregular in amplitude, after the injection. The irregularity was synchronous with muscular excitation and irregularities in the blood pressure level. It is noteworthy that the second dose, which caused a definite inversion of the pressor effect, had no such action on respiration, but instead produced a further excitation with increased rate and amplitude. Tachyphylaxis present in one physiological function is, therefore, not necessarily to be expected in others.

Discussion. While our study was in progress, reports appeared of similar studies by Hauschild and Jacobsen and collaborators (4, 5, 6, 7, 8). Both groups of investigators found *d*-methyl benzedrine more active as a psychic stimulant than was the non-methylated compound. These results later were confirmed by Golla, Blackburn and Graham in England (9). Both the British and Danish investigators emphasized that methyl benzedrine is an effective analeptic in anesthetized animals. The methyl compound is extremely weak on the gastrointestinal tract and circulation, as shown by Domenjos and Fleisch (10), Reiser (11), and Larsen (12), as well as by others quoted above.

As a result of this predilection of methyl benzedrine for the central nervous system, the *d*-isomer has been widely studied and used in man as an analeptic or central stimulant. Jacobsen (7) administered a series of these compounds to about 100 male subjects under carefully controlled conditions and observed stimulation from the methyl derivative comparable to that from benzedrine itself. When *d*-methyl benzedrine ("pervitin") was given by mouth to patients, there was general agreement among Seifert (13), Pullen (14), and Franke (15) that only small effects on blood pressure, pulse, or respiration occurred unless doses exceeding 10 mgm. were administered. Fifteen mgm. appeared to produce about 30 to 40 millimeter rises in systolic and diastolic blood pressures, lasting upwards of 6 or 7 hours. Seifert claimed that this latter dose markedly increases the respiratory volume and quotient, but does not change the basal metabolism. In view of Myerson's report (16) that benzedrine administered repeatedly to patients produced a marked polycythemia, Seifert's claim is of special interest that no such effect was observed with "pervitin." That there is no increase in the basal metabolic rate in man with moderate doses of up to 12 mgm. was confirmed by Bussemaker and Sonnenberg (17). In addition, these authors reported that the efficiency of muscular contractions was not improved, as judged by the energy utilized in performing a fixed amount of work. Other studies on muscular efficiency, carried out by Heyrodt and Weissenstein (18) and Szakall and collaborators (19, 20), confirmed that the general nature of the muscular contraction process is not altered by this drug. Apparently "pervitin" diminishes the perception of fatigue, thereby permitting the subjects to exert themselves beyond the normal physiological limits and to put out a greater total amount of work. Such an action might be of some use as an emergency measure, but it could only be harmful if used excessively as a means of exceeding the

normal physiological limitations, since the efficiency of doing work is not increased by the "pervitin," nor is the character of the muscle recovery process changed. The drug simply changes the neural physiological ceiling mechanism, which protects the organism from overstrain.

Reports on the effects of the drug in the psychological sphere have been made by many clinicians (21–30). In general, these agree that the compound elevates the mood, brings about an increased flow of ideas and an increased ability to perform mental tasks, although not necessarily increased accuracy or precision of thought. The ability to concentrate is not improved, and if the excitation is still present at the normal sleeping period, insomnia is apt to be experienced. The main uses for "pervitin" would seem to be to relieve depression in very carefully selected patients, to induce feelings of well being post-operatively, to stimulate interest in hypotonic or asthenic states, and as an analeptic to shorten the recovery from general or local anesthetic drugs.

It is interesting that a number of the clinical reporters comment on the fact that they have never observed addiction to this drug, although they seem to believe that there is some possibility of such an action. Use of the drug is not without danger. Kramer (31) has reported 9 cases of severe degrees of overstimulation in psychopathic patients, in whom various types of depression were changed disadvantageously to hypomanic or full maniacal states. Püllen (32) reports 2 cases with adverse effects, a single dose of 60 mgm. by mouth having produced unpleasant degrees of excitation and insomnia, and circulatory collapse, which was alarming for approximately half an hour. These reactions are probably the result of overdosage, since the various reporters are in general agreement that the optimum therapeutic dose is 3 to 6 mgm. by mouth, preferably administered in the morning. If the drug is given in the afternoon, it is apt to interfere with sleep that night. Higher doses can be administered in patients who have proved not to respond adequately to lower doses but, when doses of 15 mgm. are given, circulatory changes become manifest, and 30 mgm. or more may lead to unpleasant and possibly dangerous reactions.

CONCLUSIONS

1. Ten phenyl-isopropyl-amines with secondary and tertiary amino substitutions have been studied for effects on the central nervous system, circulation, and respiration.

2. In rats, dextro methyl benzedrine appears to be slightly more active as a cerebral excitant than dextro benzedrine, as indicated by the threshold stimulant dose and the duration of action. It does not, however, produce as intense an action as, or a greater total effect at the optimal dosage level, than does the unmethylated compound.

3. The circulatory effects of these compounds are minimal. Methyl benzedrine produces only a slight increase in blood pressure, while the other compounds produce mainly no effect at all, or depressor responses, when high doses are used. Tachyphylaxis makes it difficult to obtain more than a single pressor response to any of these compounds.

4. Respiration is stimulated by methyl benzedrine in both amplitude and

rate. The increase in respiratory volume precedes the development of muscular excitation, but the latter helps to maintain a greater respiratory activity.

5. Levo-methyl benzedrine is much less active than the dextro- or racemic-mixture as indicated by amount of compound required for stimulation. However, *when adequate doses are used, it produces almost as great a peak effect as do* the other two isomers. Its duration of action so greatly outlasts these other agents that the total stimulation produced is greater than that from any other compound. Effects persisting 21 hours occurred with a dose of 128 mgm. per kilogram, which caused no fatalities. Whether the prolonged action of the levo *isomer might have clinical value remains to be ascertained.*

6. Since the central stimulant action of dextro methyl benzedrine is as good as that of benzedrine, or probably greater, and the circulatory action is smaller, this would seem to be the drug of choice where increased activity of the central nervous system is clinically desired.

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THE ACTION OF ADRENALINE UPON THE ATROPINE-ACETYL CHOLINE REVERSAL PHENOMENON

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Bülbring and Burn (1) have just published the results of some experiments supporting the conclusion, previously reached by Marrazzi (2), that adrenaline depresses ganglionic transmission.

They employed several technics in their experiments, only one of which is relevant for the purpose of this note. It consisted in administering acetyl-choline in the atropinized, spinal cat (apparently adrenalectomized in some cases and not in others) during an infusion of adrenaline. They found that "the pressor effect of acetyl choline was always either depressed or abolished." The tracing they give shows a slight rise of pressure followed by a slight fall.

We also, in experiments similar to those of Bülbring and Burn, had obtained results leading to the same conclusion. The only justification for offering them for publication now is that they are in a somewhat different form and involve a few details not contained in the paper of Bülbring and Burn.

Cats were employed. Under ether anesthesia the cord was transected at the level of the atlas vertebra and the brain was destroyed. The adrenals were not removed. A Starling pump was used to maintain artificial respiration and heparin was used as an anti-coagulant.

After atropinization an infusion of adrenaline, sufficient to maintain only a moderate rise of blood pressure, was begun. A test injection of adrenaline from a syringe was then made to insure that a further rise of pressure could be elicited. This done, an injection of a large dose of acetyl choline was made. Instead of the rise of pressure which occurs when acetyl choline is injected into the atropinized spinal cat, a moderate and transient fall (which will be discussed below) occurred. A few minutes later the injection of adrenaline produced the same effect as when first tested. The infusion of adrenaline was then stopped. When the blood pressure had returned to the initial level another injection of acetyl choline caused an almost pure rise in blood pressure (fig. 1, *A* to *G*).

The results of a similar experiment are shown in fig. 2, *A* to *H*. In this case, however, when the acetyl choline injection was made at *H* some adrenaline influence still existed which had disappeared when the injection was repeated at *P*. The last portion of this experiment is a repetition of the *C-D-E* section.

While there is no reason to believe that the pressor substance of the pituitary gland would influence the atropine-acetyl choline phenomenon, several experiments were carried out to see what did happen. The preparation described by Stehle and Fraser (3) was used. These experiments showed that the pressor effect of acetyl choline occurs as it does in the absence of the pressor substance. They also provide a possible explanation for the depressor effect of the acetyl

choline in the atropine-adrenaline-acetyl choline experiments. It may be seen in fig. 2, at *M* that during the infusion of the pressor substance the pressor effect of acetyl choline was preceded by a depressor effect. In this experiment the blood pressure was quite high. In the experiment shown in fig. 1 (*K*, *L*), in which the blood pressure was low, there was no preliminary fall of blood pressure. It would appear, therefore, that the pressor action of acetyl choline, administered *after atropine, is probably the resultant of vascular constrictor and vascular* dilator actions occurring simultaneously, and that the "purity" of the pressor

Fi(
A, 5 mgm.
aline per mini
hydrochloride

gm. adrenaline (injection).

effect depends upon the blood pressure level. This depressor component we regard as responsible for the fall of blood pressure in the atropine-adrenaline-acetyl choline experiments.

It may be that atropinization is never complete in the sense that all of the dilator action of acetyl choline is abolished. Either the acetyl choline can break through the atropine barrier to some extent or some vessels remain which are not affected by atropine In the latter case such vessels would represent only a small section of the whole vascular cross-section.

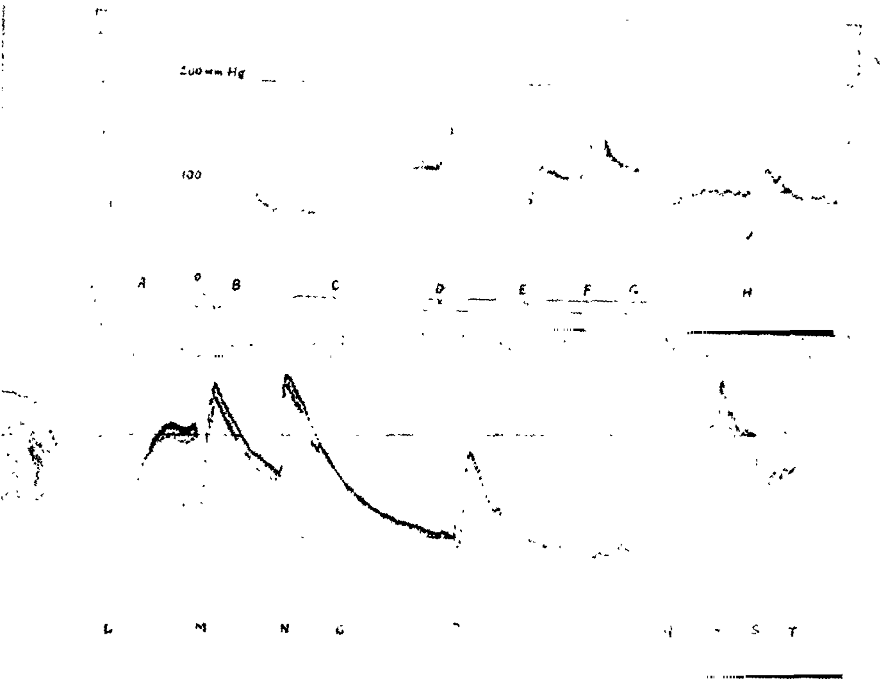

FIG. 2. SPINAL CAT, MALE, 3.4 KGM. TIME RECORD IN MINUTES

A, 5.0 mgm. atropine sulfate (injection); B, 5.0 mgm. acetyl choline (injection); C, intravenous infusion of 0.02 mgm. adrenaline per minute begun; D, 0.02 mgm. adrenaline (injection); E, 5.0 mgm. acetyl choline hydrochloride (injection); F, 0.02 mgm. adrenaline (injection); G, adrenaline infusion stopped; H, 5.0 mgm. acetyl choline (injection); L, intravenous infusion of 10 pressor units per minute of the pressor hormone of the pituitary gland begun; M, 5.0 mgm. acetyl choline hydrochloride (injection); N, 0.02 mgm. adrenaline (injection); O, pituitary infusion stopped; P, 5.0 mgm. acetyl choline hydrochloride (injection); Q, intravenous infusion of 0.02 mgm. adrenaline per minute begun; R, 0.02 mgm. adrenaline (injection); S, 5.0 mgm. acetyl choline hydrochloride (injection); T, adrenaline infusion stopped.

FIG. 3. SPINAL CAT, MALE, 3.1 KGM. TIME RECORD IN MINUTES

A, 5.0 mgm. atropine sulfate intravenously; B, same as A; C, intravenous infusion of 0.02 mg. per minute of racemic arterenol begun; D, 0.02 mgm. adrenaline (injection); E, 5.0 mgm. acetyl choline hydrochloride (injection); F, 0.02 mgm. adrenaline (injection); G, arterenol infusion stopped; H, 5.0 mgm. acetyl choline hydrochloride (injection).

An alternative explanation for the phenomenon may be suggested. It is possible that the block imposed by adrenaline in the ganglia may affect only the ganglionic terminations of sympathetic vasoconstrictor fibers leaving the terminations of sympathetic vasodilator fibers unaffected.

Several experiments were carried out in which arterenol was substituted for adrenaline. They showed the two substances to be indistinguishable in regard to the action in question (fig. 3).

SUMMARY

1. During intravenous infusion of adrenaline or arterenol in the atropinized spinal cat, the pressor response to a large dose of acetyl choline is abolished and a transient depressor response noted.

2. During intravenous infusion of posterior pituitary extract under similar conditions the pressor response to acetyl choline is not abolished but may be preceded by a transient depressor response when the blood pressure level is high.

3. These results are in agreement with those of Marazzi and Bülbring and Burn, which show that adrenaline depresses ganglionic sympathetic transmission. Arterenol also exerts a similar action.

4. Possible mechanisms of the transient depressor effects of a large dose of acetyl choline as observed in the above experiments are discussed.

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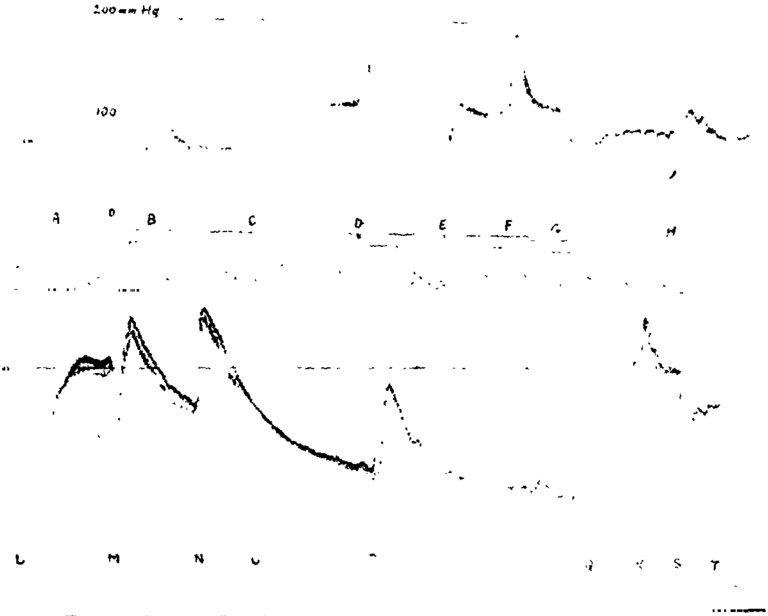

FIG. 2. SPINAL CAT, MALE, 3.4 KGM. TIME RECORD IN MINUTES

A, 5.0 mgm. atropine sulfate (injection); B, 5.0 mgm. acetyl choline (injection); C, intravenous infusion of 0.02 mgm. adrenaline per minute begun; D, 0.02 mgm. adrenaline (injection); E, 5.0 mgm. acetyl choline hydrochloride (injection); F, 0.02 mgm. adrenaline (injection); G, adrenaline infusion stopped; H, 5.0 mgm. acetyl choline (injection); L, intravenous infusion of 10 pressor units per minute of the pressor hormone of the pituitary gland begun; M, 5.0 mgm. acetyl choline hydrochloride (injection); N, 0.02 mgm. adrenaline (injection); O, pituitary infusion stopped; P, 5.0 mgm. acetyl choline hydrochloride (injection); Q, intravenous infusion of 0.02 mgm. adrenaline per minute begun; R, 0.02 mgm. adrenaline (injection); S, 5.0 mgm. acetyl choline hydrochloride (injection); T, adrenaline infusion stopped.

FIG. 3. SPINAL CAT, MALE, 3.1 KGM. TIME RECORD IN MINUTES

A, 5.0 mgm. atropine sulfate intravenously; B, same as A; C, intravenous infusion of 0.02 mg. per minute of racemic arterenol begun; D, 0.02 mgm. adrenaline (injection); E, 5.0 mgm. acetyl choline hydrochloride (injection); F, 0.02 mgm. adrenaline (injection); G, arterenol infusion stopped; H, 5.0 mgm. acetyl choline hydrochloride (injection).

In the first the craniostomy was done under light ether anesthesia, puncturing with a glass needle and recording the temperature in the unanesthetized animal in the second stage. This resulted 15 minutes after the thermopuncture, in a

TABLE 1

RABBIT NO.	TEMPERATURE NORMAL	MINUTES OF INHALING BENZOL					
		10'	20'	30'	40'	50'	60'
54	39 5	37.3	35.0	34.0	33.2		
55	39.0	38.2	36.0	35 8	35.0	34.2	33.0
57	39.0	38.3	37.3	35.7	35 2		
59	39.3	38.5	37.3	35.3	34.8		
60	39.1	37.9	36.8	35 5	34.6	33.8	33.1
Average.	39 1°	38 3°	36.6°	35.3°	34 6°	34.0°	33.1°

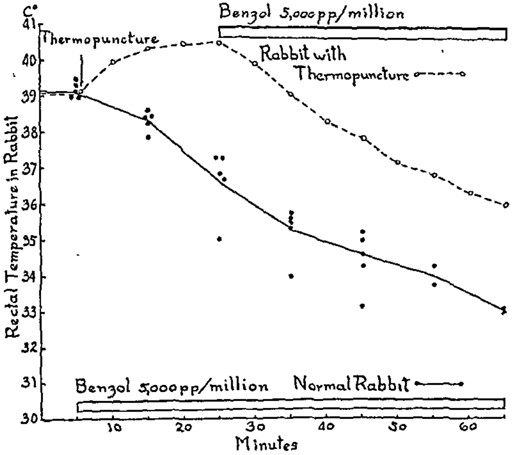

FIG. 1

average rise in the rectal temperature to 40.5°C. The temperature fall with the benzol was progressive and slow, even though not as intense as when normal animals were used under the same concentration of inhalation.

It is well known that, with ether anesthesia in the rabbit, the temperature falls, and this we have verified, although falls of only 1.7°C. were obtained in the

THE ACTION OF BENZOL ON CERTAIN CENTRAL NERVOUS REGULATING MECHANISMS

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For studies on central actions of benzol the method of inhalation has been employed because it represents the most common manner of entrance to the body in industrial hazards, and at the same time, as has previously been reported (1, 2) facilitates control of the concentration in the body. In the present study attention has been given to the following fields of action: sympathetic, temperature regulation, shivering and panting.

SYMPATHETIC. Sympathomimetic reactions are found in animals submitted to benzol inhalation. In the rabbit an intense mydriasis was observed, the pupillary diameter increasing from 5.3–5.8 mm. to 6.8–7.2 mm.; in these same animals gastrointestinal paralysis was usual and was followed by passive abdominal dilatation after a long period of inhalation; also piloerection has been found on some occasions, although this is more frequent in the cat. Such findings served for orientation in the first article (1), by associating them with those obtained by Karplus and Kreidl (3) in the stimulation of the walls of the third ventricle in the hypothalamic region, those of Bard (4) describing the sham rage produced by excitation of the posterior area, a phenomenon mediated through the sympathetic, and those of Ranson (5) observed with faradic stimulation of this region. However, even when the sympathomimetic character predominates, with small doses of benzol there apparently is also some cholinergic excitation, such as increase in tone of the isolated intestine and salivation.

TEMPERATURE. The first bibliographic mention of the changes in the temperature provoked by benzol appears to be that of Chassevant and Garnier (6) who, from a general point of view, considered the benzene ring as a hypothermic agent. Lehmann (7) and his collaborators found slight falls in temperature in cats exposed to benzol. Pruneda, in a personal communication, mentioned the sensation of intense cold observed in some cases of industrial intoxication with benzol. The values obtained for the rectal temperature of rabbits submitted to inhalation of benzol in concentration below 5,000 parts per million of air (.015 grams/liter), are given in table 1.[2]

The average shows approximately a fall of 1°C. in the rectal temperature each 10 minutes of inhalation, descending from 39.3° to 33.1°C. at the end of an hour, when the room temperature varied in the recording period between 18° and 23°C. In rabbits with hyperthermia produced by Richet's technique with hypothalamic puncture (rabbits no. 56, 58 and 61) the experiment was done in two stages.

[1] Fellow of El Colegio de México and The Rockefeller Foundation (Yale University, Dept. of Pharmacology).

[2] Benzol (Benzene) Mallickrodt, control EK, was employed.

In the first the craniostomy was done under light ether anesthesia, puncturing with a glass needle and recording the temperature in the unanesthetized animal in the second stage. This resulted 15 minutes after the thermopuncture, in a

TABLE 1

RABBIT NO.	TEMPERATURE NORMAL	MINUTES OF INHALING BENZOL					
		10'	20'	30'	40'	50'	60'
54	39.5	37.3	35.0	34.0	33.2		
55	39.0	38.2	36.0	35.8	35.0	34.2	33 0
57	39.0	38 3	37.3	35.7	35.2		
59	39.3	38 5	37.3	35.3	34 8		
60	39.1	37.9	36.8	35.5	34.6	33.8	33 1
Average.	39.1°	38 3°	36.6°	35.3°	34 6°	34 0°	33.1°

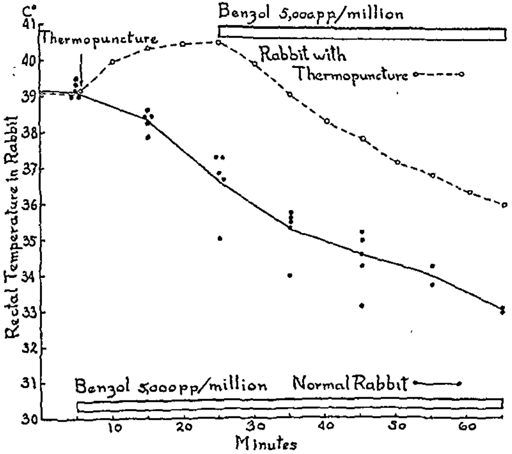

FIG. 1

average rise in the rectal temperature to 40 5°C. The temperature fall with the benzol was progressive and slow, even though not as intense as when normal animals were used under the same concentration of inhalation.

It is well known that, with ether anesthesia in the rabbit, the temperature falls, and this we have verified, although falls of only 1.7°C. were obtained in the

first 40 minutes. With the barbiturates, chloral and chloroform, the fall in temperature is greater; furthermore, Molitor and Pick (8) have demonstrated, that while urethane stimulates the respiratory center, barbital paralyzes it, which actions, with the foregoing, can interfere with the results and which are avoided by the two-stages operation.

SHIVERING. "The generalized involuntary trembling of the body muscles, convulsive and rhythmic in character, accompanied by a sensation of cold" (Richet 1893) appears a few minutes after the commencement of benzol inhalation in the experimental animals, as one of the salient characteristics.

The shivering in rabbits breathing an atmosphere containing a benzol concentration of less than 5,000 pp./million, is progressively intensified until the convulsive muscular movement is permanent and only begins to decline in these animals at the end of 60 minutes. Beattie (9) has also found a similar course of events in the reflex shivering of hypothalamic integration, which may disappear after some time, giving way to general muscular relaxation. In the cat

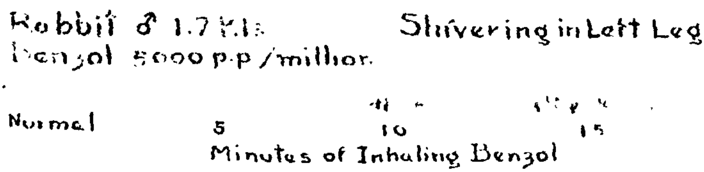

FIG. 2

and dog its character is similar, even though in the latter the required concentration of benzol is higher.

Along with the appearance and the development of the shivering in animals which inhale benzol, another motor phenomenon, panting is provoked, and this behaves in a parallel manner with progressive sectioning of the brain stem.

PANTING. In dogs and rabbits similar behavior was repeatedly found under the inhalation of benzol. The character of the respiratory movements, studied by direct observation of the animal as well as by means of a pneumogram in the dog, shows a diminution in amplitude principally after the first 10 minutes and the panting in the dog as in the rabbit is very marked.

In cases in which the records were obtained on dogs subjected to narcosis with chloral-morphine, the respiratory rate was reduced by 7 or 8 per minute, as can be seen in fig. 3; with the inhalation of benzol, records of an average of 81 respirations per minute could be obtained with 30 minutes of exposure to an atmosphere of 15,000 pp./million; the panting appears with equal intensity in animals whose respiratory centers had been depressed pharmacologically as in morphinized dogs, in which the initial figures, instead of being 15–20 per minute as normally, are 6–8 per minute.

The behavior of the shivering and panting along the different sections of the brain stem was then studied.

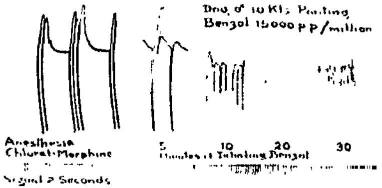

Fig. 3

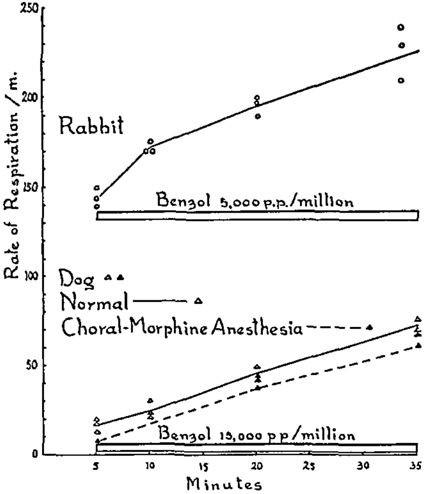

Fig 4

Technique. Rabbits of 1.3–3 0 kg and cats of 2 0–3.8 kg (the latter especially used for *the lower brain stem sections because of their greater resistance to operations)*, under ether anesthesia, were tracheotomized and given artificial respiration. Both carotids were ligated and craniostomies in the parietal regions or high laminectomies, according to case, were performed The sections were made with a thin but rather blunt spatula of aluminium,

always with the animal panting by benzol, and the direction and localization of the cut were verified at autopsy. Hemostasis was accomplished as ordinarily with muscle and hot saline. All the experiments were acutely performed.

RESULTS. *Decortication.* (*animals 63, 64, and 66*). In the cases where the removal of both hemispheres of the cortex was done away from the midline, there was no alteration in the panting or shivering.

Section below the tentorium by the Sherrington technique (*animals no. 66 and 67*). After the double craniotomy the brain stem was rapidly cut in order to avoid shock and hemorrage, following the tentorium behind the posterior corpora quadrigemina and mammillary bodies but above the pons. After the section, suppression of the panting and shivering was expected, but in no case did either disappear, although their intensity decreased very slightly.

Section similar to the above but with the removal of the whole cerebellum (*animal no. 69*). In this case, in the first part of the experiment, the plane of the tentorium behind the posterior corpora quadrigemina and mammillary bodies

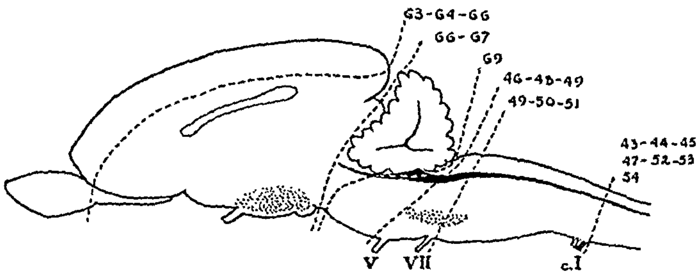

FIG. 5. SCHEME OF THE BRAIN STEM TRANSECTIONS IN THE RABBIT

was followed, with later total removal of the cerebellum; after this, the animal did not present any change in the panting or in the shivering.

Section in the upper part of the pons immediately above the apparent exit of the trigeminal nerve (*animals no. 46, 48 and 49*). In this case, after the ablation of all the superior part of the mesencephalon, diencephalon and telencephalon only a slight weakening of the motor phenomena was encountered, but the picture of panting and shivering was perfectly clear.

Section through the level of the striae acousticae and immediately behind the apparent exit of the facial nerve (*animals no. 49, 50 and 51*). In this case both phenomena disappeared completely after the section.

Section through the medulla at the first cervical nerve, with the removal of the whole brain stem above (*animals no. 43, 44, 45, 47, 52, 53, and 54*). This was done primarily to differentiate any inferior medullary integration from the phenomena; in none of these "spinal animals" could the shivering or panting responses be obtained.

DISCUSSION. The shivering which appears with benzol does not depend on the atmospheric temperature, or on a reflex mechanism through the vagus, be-

cause it occurs even when the animal is in a room at 40°C. (rabbits no 71, 72, and 73) and when the vagus nerves are cut (rabbits no 72 and 74, and cat no 75). In many cases shivering under benzol inhalation appears before any alteration in the rectal temperature, which seems to indicate that it is concerned with a direct action on some heat regulating center. Dworkin (10) has found in rabbits that shivering still occurs after complete transection about 2 mm. above the calamus scriptorius, although there is evidence that after the transection through or below the diencephalon, the threshold for this reflex is raised. About the level of the calamus, typical shivering was replaced by a less integrated, less generalized and less intense form of muscular response as clonic spasm and incoordinated tremors; the intensity of muscular response to cooling is diminished gradually as the medulla oblongata is progressively sectioned, but there is no abrupt disappearance of shivering. Contrary to this opinion are the ideas of Miller (11) who says that an animal with hypothalamus damaged cannot shiver, but no artificial stimulation or experimental destruction of the hypothalamus has yet confirmed this.

Anrep and Hammouda (12) have studied the panting behavior and have considered anesthesia, temperature, blood carbon dioxide and the anoxemia provoked. Hammouda (13) later following transections of the brain, and heat directly applied to the third ventricle, believed that the integration of panting is located in the optic thalamus although, if his sections are examined carefully, part of the lesions are seen to have affected various hypothalamic nuclei. Keller (14) disagrees with this opinion because panting can occur even if the diencephalon is not intact. Clark, Magoun, and Ranson (15) believe that the motor mechanism responsible for coordinated panting movements is not destroyed by thalamic or hypothalamic lesions. Our researches agree with the recent work of Lumdsen (16) who localizes motor respiratory centers around the level of the striae acousticae and with Pitts (17) who, employing electrical stimulation instead of transection of the brain stem, touched on similar inhibitory systems.

CONCLUSIONS

Benzol exercises a specific central action on a zone of integration of panting and shivering; this zone may often be influenced by impulses mediated through the hypothalamus, as in the control of the body temperature. From the study of transections in acute experiments, it is deduced that the zone of integration is contained in the inferior portion of the pons and the middle portion of the medulla oblongata, possibly in the reticular formation, of great motor importance in these mammals.

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STUDIES ON THE FATE OF NICOTINE IN THE BODY

III. ON THE PHARMACOLOGY OF SOME METHYLATED AND DEMETHYLATED DERIVATIVES OF NICOTINE

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The present communication presents a comparison of (A) the toxicity, (B) the blood pressure effects, and (C) the fate in the animal organism of nicotine and certain derivatives of nicotine (fig. 1). Of these, the *N*-methyl derivatives are of particular interest. It is well established that pyridine and nicotinic acid are detoxified in the animal body, at least in part, by methylation. Accordingly, the possibility that nicotine might be detoxified in a similar manner seemed worthy of consideration.

A. Toxicity. Excepting nicotine, available information concerning the toxicity of the above substances for mammals is slight. Macht and Davis (1), using racemic nornicotine, concluded that for some species of mammals it is less toxic and for others more toxic than the corresponding racemic mixture of nicotine. Of the methyl derivatives listed (fig. 1), toxicological data have been previously presented only for dimethyl nicotine, by Crum-Brown and Fraser (2) and Loos (3). These authors found that dimethyl nicotine is much less toxic than nicotine, but the number of animals used in the tests was too small to permit an exact comparison.

In the toxicological experiments reported here, male albino mice and rabbits were used. For the intraperitoneal injections in mice, volumes varying from 20–30 cc. per kgm. body weight were employed. Concentrations for intravenous injection were adjusted to make a constant injection volume of 1.5 cc. per kgm. *and this was administered at the rate of 0.1 cc. per second. Neutral unbuffered* solutions were used in each case.

The results of these experiments are reported in table 1. For comparative purposes, since the molecular weights of these substances differ widely, all doses and LD_{50} values are expressed in terms of millimols per kgm. of body weight. On this basis, by intraperitoneal administration to mice, *l*-nornicotine[1] was found to be about one-half as toxic as *l*-nicotine, monomethyl nicotinium iodide twice as toxic, isomonomethyl nicotinium iodide one-thirty-third as toxic and dimethyl nicotinium di-iodide one-thirty-sixth as toxic.

The intravenous toxicity tests reveal a somewhat different picture (table 1). The *relative* toxicity of nornicotine and monomethyl nicotinium iodide is markedly increased, making them 2 and 14.5 times more toxic respectively than nicotine. Isomonomethyl nicotinium iodide and dimethyl nicotinium di-iodide

[1] Kindly furnished to us by Dr. R. C. Roark, United States Department of Agriculture, Bureau of Entomology and Plant Quarantine.

intravenous toxicities were not determined on a sufficient number of animals to obtain accurate quantitative results, but it is apparent that both are much less toxic than nicotine and that the *relative* toxicity of the isomonomethyl derivative is somewhat increased.

The differences noted in *relative* toxicity of these compounds from the two different routes of administration are apparently a result of differences in their rate of absorption. This in turn may be related to their degree of ionization. It is well known that the free base of nicotine is much more readily absorbed from extravascular sites of administration than is its ion. As will be seen below, at blood pH about 16% of nicotine exists as the free base. *Compared to this, all of the methyl derivatives of nicotine used here are virtually completely ion-*

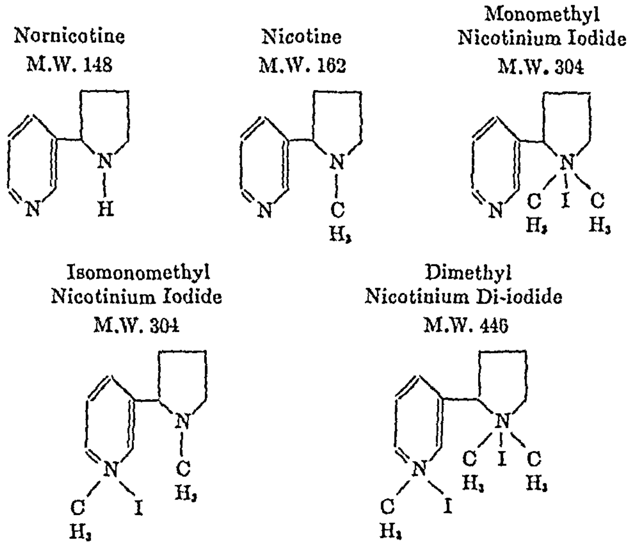

FIG. 1

ized and hence it might be expected that their *relative* toxicity compared to nicotine would be greater on intravenous injection. The failure of isomonomethyl and dimethyl nicotine compared to monomethyl nicotine to show marked differences in toxicity by these two routes of administration may be due to their inherent low toxicity.

Nornicotine in this instance well illustrates the greater absorbability of the free base following intraperitoneal injection. At room temperature the pK_1 for nicotine was found by electrometric titration to be 8.07, while that for nornicotine was 9.09. From this it may be calculated that at a blood pH of 7.35, 16% of nicotine exists as the free base compared to 1.7% for nornicotine. It is believed that this difference in the ratio of salt to base is responsible for the observation that nornicotine is only one-half as toxic as nicotine by intraperitoneal

injection in mice, whereas by intravenous injection in rabbits it is twice as toxic as nicotine. To meet the possible objection that the difference in *relative* toxicity of nornicotine to nicotine by the two routes of administration used here is due to difference in species of animals used, a comparison of their intraperitoneal

TABLE 1

Acute toxicity

SUBSTANCE	SITE OF INJECTION	DOSE	ANIMAL	NUMBER OF ANIMALS	FATALITIES	LD_{50}	AVERAGE TIME OF DEATH	LD_{50} IPM[2] / LD_{50} IVR
		millimols per kgm.				*millimols per kgm.*	*minutes*	
l-nornicotine	Intraperitoneal	0.135	Mouse	40	15	0.147	8.1	7.2
	Intraperitoneal	0.169	Mouse	40	38		5.8	
l-nicotine	Intraperitoneal	0.062	Mouse	40	19	0.0635	2.7	1.6
		0.068	Mouse	40	23		3.1	
Monomethyl nicotinium iodide	Intraperitoneal	0.031	Mouse	40	19	0.0308	9.7	11.6
	Intraperitoneal	0.037	Mouse	40	27		10.2	
Isomonomethyl nicotinium iodide	Intraperitoneal	1.85	Mouse	40	13	2.10	9.7	*ca* 4
	Intraperitoneal	2.16	Mouse	40	22		10.7	
Dimethyl nicotinium di-iodide	Intraperitoneal	2.16	Mouse	40	13	2.29	16.5	<2.5
	Intraperitoneal	3.08	Mouse	40	36		14.1	
l-nornicotine	Intravenous	0.0204	Rabbit	10	5	0.0204		
l-nicotine	Intravenous		Rabbit	35		0.0386[1]		
Monomethyl nicotinium iodide	Intravenous	0.00185	Rabbit	10	1	0.00265		
	Intravenous	0.00308	Rabbit	10	7			
Isomonomethyl nicotinium iodide	Intravenous	0.308	Rabbit	3	0	0.463–0.617		
	Intravenous	0.463	Rabbit	3	1			
	Intravenous	0.617	Rabbit	3	2			
Dimethyl nicotinium di-iodide	Intravenous	0.617	Rabbit	2	0	>0.925		
	Intravenous	0.925	Rabbit	2	0			

[1] Taken from Haag, H. B., J. Lab Clin. Med, 25: 610, 1940.

[2] Ratio of intraperitoneal toxicity in mouse to intravenous toxicity in rabbit.

toxicities to rabbits was made. The intraperitoneal LD_{50} of nicotine for rabbits is about 14 mgm. per kgm. Accordingly the effects of 15 mgm. per kgm. of nicotine and of the molecular equivalent of this of nornicotine (13.7 mgm. per kgm.) were tested following intraperitoneal injection into rabbits. In this test, nicotine killed 6 out of 10 animals while nornicotine killed only 1 out of 10.

An analysis of the average time of death (table 1) following intraperitoneal injection also supports the observation that nicotine is more rapidly absorbed than are the derivatives of it studied here.

It may be concluded from these experiments that the *relative* toxicity of substances of this type may differ markedly according to the method of administration.

B. BLOOD PRESSURE. Excepting nicotine, no reference to the blood pressure effects of these compounds has come to the authors' attention. The relative blood pressure effects of these substances were therefore determined following their intravenous injection on four atropinized dogs anesthetized with a solution of diallylmalonylurea and ethyl carbamate (Dial-Ciba with urethane). Again comparing all results with nicotine, the pressor potency, as determined from the peak of pressure, of nornicotine is about one-twelfth, and that of monomethyl nicotine about two-thirds that of nicotine. This difference in the pressor potency of nicotine and nornicotine is in line with the observations of Barger and Dale (4), who found in a series of sympathomimetic amines that insertion of a methyl group into the primary amine increases the blood pressure activity 5–10 times.

Isomonomethyl nicotine and dimethyl nicotine caused a fall in blood pressure, the threshold dose being about 0.012 millimols per kgm. in both instances. With moderate doses, decreases in blood pressure were transient.

C. FATE IN ANIMAL ORGANISM. In a recent investigation (5) on the fate of nicotine in the dog, we found that only about 10% of administered nicotine was excreted unchanged in the urine. The remainder was not converted to 1-methyl pyridinium hydroxide, nicotinic acid, nicotinuric acid or trigonelline, although it did appear in the nicotinic acid fraction of the urine as determined by the method of Perlzweig *et al.* (6) both before and after acid hydrolysis. An apparent urinary end product of nicotine detoxication that is characterized by its formation of a rose color with cyanogen bromide was described, but identification of this substance was not accomplished.

Since detoxication by methylation is known to occur for pyridine and nicotinic acid, investigation of the fate of the methyl derivatives of nicotine in the animal organism was a prime purpose of this study. In addition, the possibility that nicotine might be demethylated in the course of detoxication was examined by comparing the fate of nornicotine with that of nicotine.

This study was performed on dogs and the experimental techniques employed were identical with those previously described (5), with the exception that the determination for 1-methyl pyridinium hydroxide was omitted. Thus 24 hour collections of urine from 3 dogs were analyzed, before and after administration of the individual nicotine derivatives, for trigonelline, nicotinic acid fraction before and after acid hydrolysis and for the presence of any substance yielding a rose color with cyanogen bromide. Results of these studies are presented in table 2.

From these data it is apparent that, unlike nicotine, none of the methyl derivatives of nicotine or their detoxication products appeared among the substances

or groups of substances for which analyses were made, nor did their administration lead to the presence in the urine of any substance forming a rose color on addition of cyanogen bromide. We are, therefore, brought to the conclusion that either methylation is not a step in the detoxication of nicotine, or if it is, there must be more than one end product of nicotine detoxication.

Nornicotine on the other hand increased the magnitude of the nicotinic acid fractions to a degree equal to that previously observed by us following administration of identical quantities of nicotine. Likewise a reddish color appeared in the urine on addition of cyanogen bromide. In the previous experiments with nicotine, it was shown that nicotine excreted unchanged in the urine could not have been responsible for the rose color produced by cyanogen bromide since this combination results in a greenish yellow hue. However, when similar tests were made with nornicotine, a deep red color was obtained on addition of cyanogen bromide. Analyzed spectrophotometrically, this color showed a minimum transmittance at 540 mμ.

TABLE 2

Influence of some methylated and demethylated nicotine derivatives on the excretion of certain pyridine derivatives by the dog

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13
N.A.F.U., mgm.	0.40	0.35	3 31	0 43	0.37	0 42	0.33	0 33	0.28	0.30	0.33	0.35	0.39
N.A.F.H., mgm.	1.00	0.92	4 26	1 15	0.98	0.83	0 83	0.84	0 94	0.81	0.81	0.80	0.91
Trigonelline, mgm.	8.8	8.7	9.7	8.2	8.5	8.2	8 2	8 9	9.0	8.4	9.1	8.5	9.6

Symbols: N.A.F.U—Nicotinic acid fraction unhydrolyzed. N.A.F H—nicotinic acid fraction hydrolyzed. All figures are expressed as the average of results from 3 dogs On day 3, nornicotine, equivalent to 28 7 mgm. of nicotine was administered to each dog. On day 6, dimethyl nicotine, on day 9, isomonomethyl nicotine and on day 12, monomethyl nicotine were similarly administered

Two questions arise from these observations, namely, is sufficient nornicotine excreted unchanged following its administration to account for all of the red color appearing in the urine following addition of cyanogen bromide and is the unknown substance that we have described in urine following nicotine administration nornicotine?

Based on the red color developed by the nornicotine-cyanogen bromide reaction a colorimetric method for the quantitative determination of nornicotine was developed.[2] Two aliquots of the urine obtained following administration of nornicotine were then analyzed as follows: On one aliquot, nornicotine was determined directly, following decolorization of the urine by the procedure described by Perlzweig *et al.* (6). By this procedure it was found that of the 28.7 mgm. of nornicotine administered to each dog, an average of 1.9 mgm. was apparently excreted unchanged. An ether extract was made of the second aliquot and on analysis for nornicotine an average of 1.8 mgm. was found. Analyzed spectrophotometrically, the red color obtained by reacting this ether-soluble

[2] To be published.

An analysis of the average time of death (table 1) following intraperitoneal injection also supports the observation that nicotine is more rapidly absorbed than are the derivatives of it studied here.

It may be concluded from these experiments that the *relative* toxicity of substances of this type may differ markedly according to the method of administration.

B. Blood pressure. Excepting nicotine, no reference to the blood pressure effects of these compounds has come to the authors' attention. The relative blood pressure effects of these substances were therefore determined following their intravenous injection on four atropinized dogs anesthetized with a solution of diallylmalonylurea and ethyl carbamate (Dial-Ciba with urethane). Again comparing all results with nicotine, the pressor potency, as determined from the peak of pressure, of nornicotine is about one-twelfth, and that of monomethyl nicotine about two-thirds that of nicotine. This difference in the pressor potency of nicotine and nornicotine is in line with the observations of Barger and Dale (4), who found in a series of sympathomimetic amines that insertion of a methyl group into the primary amine increases the blood pressure activity 5–10 times.

Isomonomethyl nicotine and dimethyl nicotine caused a fall in blood pressure, the threshold dose being about 0.012 millimols per kgm. in both instances. With moderate doses, decreases in blood pressure were transient.

C. Fate in animal organism. In a recent investigation (5) on the fate of nicotine in the dog, we found that only about 10% of administered nicotine was excreted unchanged in the urine. The remainder was not converted to 1-methyl pyridinium hydroxide, nicotinic acid, nicotinuric acid or trigonelline, although it did appear in the nicotinic acid fraction of the urine as determined by the method of Perlzweig *et al.* (6) both before and after acid hydrolysis. An apparent urinary end product of nicotine detoxication that is characterized by its formation of a rose color with cyanogen bromide was described, but identification of this substance was not accomplished.

Since detoxication by methylation is known to occur for pyridine and nicotinic acid, investigation of the fate of the methyl derivatives of nicotine in the animal organism was a prime purpose of this study. In addition, the possibility that nicotine might be demethylated in the course of detoxication was examined by comparing the fate of nornicotine with that of nicotine.

This study was performed on dogs and the experimental techniques employed were identical with those previously described (5), with the exception that the determination for 1-methyl pyridinium hydroxide was omitted. Thus 24 hour collections of urine from 3 dogs were analyzed, before and after administration of the individual nicotine derivatives, for trigonelline, nicotinic acid fraction before and after acid hydrolysis and for the presence of any substance yielding a rose color with cyanogen bromide. Results of these studies are presented in table 2.

From these data it is apparent that, unlike nicotine, none of the methyl derivatives of nicotine or their detoxication products appeared among the substances

SUMMARY

A comparison has been made of the toxicity, the blood pressure effects, and the fate in the animal organism of *l*-nornicotine, *l*-nicotine, monomethyl nicotinium iodide, isomonomethyl nicotinium iodide and dimethyl nicotinium di-iodide.

The integrity of the pyridine nitrogen appears to be essential to the high degree of toxicity of nicotine and methylated and demethylated nicotines. On the other hand, progressive methylation of the pyrrolidine nitrogen increases the toxicity, as judged by intraperitoneal administration in white mice, in the ratio of 1:2:4, for nornicotine, nicotine, and monomethyl nicotinium iodide. The ratio of LD_{50} by intraperitoneal administration in mice to the LD_{50} by intravenous administration in rabbits is respectively 7.2, 1.6 and 11.6 for the three compounds. The difference in the dissociation constants of the three bases is offered as an explanation for this phenomenon.

The pressor effect on the anesthetized dog of nornicotine and monomethyl nicotinium iodide is respectively one-twelfth and two-thirds of that of a molecular equivalent of nicotine. Isomonomethyl nicotinium iodide and diemthyl nicotinium di-iodide in similar amounts have no effect and in doses of 100 times the threshold pressor dose of nicotine have a depressor effect.

In the dog, the course of metabolism of the methylated and demethylated derivatives of nicotine appears to be unlike that of nicotine. No evidence has been found to indicate that the detoxication of nicotine in the animal organism involves either methylation or demethylation of the molecule.

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EFFECT OF CHLOROFORM AND ETHER ON THE SENSITIVITY OF MUSCLE TO ACETYLCHOLINE

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The presence of signs of stimulation of the parasympathetic nervous system during chloroform anesthesia and the absence of these during ether anesthesia suggested that chloroform may inhibit choline esterase. *In vitro* experiments show that anesthetic (1) and high concentrations (2) of chloroform and ether inhibit choline esterase to a certain extent. Up to the present *in vivo* experiments with muscle have indicated an increased choline esterase activity (decrease of acetylcholine sensitivity of muscle) by chloroform (3), and the reverse by high concentrations of ether (4). Therefore, the subject seems to be in need of further investigation.

METHOD. The principle of the method is to determine the sensitivity of the rectus abdominis of the frog to acetylcholine after treatment with various concentrations of chloroform and ether.

The rectus abdominis muscle of the frog was excised and suspended in a muscle chamber containing 20 cc. of well aerated Ringer's solution at room temperature. The pH of the Ringer's solution was 7.1. The height of contraction produced by acetylcholine bromide was registered by an isotonic lever on a kymograph. Acetylcholine was used in a concentration producing a satisfactory contraction, neither maximum nor minimum. This varied for different muscles and lay between a concentration of 0.01 to 0.1 mgm. per 100 cc. The muscle was alternately placed in acetylcholine solution for 30 seconds and in Ringer's solution for 10 minutes, until the height of the contraction to acetylcholine became constant. This height of contraction was taken as the control. In a series of such experiments it was found that the response to the same concentration of acetylcholine remained constant throughout a period of more than three hours, longer than the experiments with chloroform and ether, so that adventitious factors (fatigue, deterioration of the muscle, etc.) could be excluded.

In the experiments designed to study the effect of ether and chloroform on the response to acetylcholine, the exposures to acetylcholine were preceded by a five-minute immersion in chloroform or ether. The following concentrations were used: For chloroform 0.0025, 0.005, 0.01, 0.02, 0.04, 0.05, 0.08, 0.1, 0.12, 0.14, 0.16 cc. per cent or saturated; for ether 0.05, 0.075, 0.1, 0.2, 0.5, 1.0, 1.5, 1.8, 2.0 cc. per cent, half saturated or saturated. In order to make the experiments comparable a five-minute exposure was selected, because it was found that the effect of chloroform and ether increased with the duration of exposure.

The effect of the various measures on the response to acetylcholine was expressed in percentage of the control response.

RESULTS. *Effect of chloroform on the response of striated frog muscle to acetylcholine.* The results show that chloroform in low concentration (0.0025 to 0.03 cc. per cent) increases the response of the striated muscle of the frog to acetylcholine. Under the conditions of these experiments, the potentiation reaches its peak with a concentration of 0.01 cc. per cent. Larger concentrations reverse the effect and cause a decrease in the response to acetylcholine.

Depression of the sensitivity of muscle to acetylcholine has also been observed by Emmelin (3). Muscles immersed in concentrated solutions of chloroform (0.160 cc, per cent or more) develop contracture (table 1, fig. 1). The contracture is reversible in the early stages (reported also by Hofmann, 5; Rossi, 6;

TABLE 1

Effect of chloroform on muscle contraction from acetylcholine

CONCENTRATION OF CHLOROFORM	NUMBER OF EXPERIMENTS	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL	
		Mean	S.E.*
cc. per cent			
0.0025	6	126	±1.0
0.005	46	157	±5.1
0.010	31	164	±9.9
0.020	11	121	±5.6
0.040	9	86	±10.5
0.050	21	67	±7.8
0.080	5	37	±8.2
0.100	5	35	±11.7
0.160 to saturated	40	Contracture	

* S.E. of mean $= \sqrt{\dfrac{\Sigma (\Delta)^2}{N(N-1)}}$.

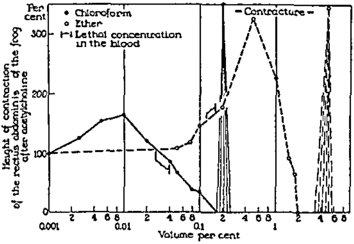

FIG. 1. HEIGHT OF CONTRACTION OF THE RECTUS ABDOMINIS OF FROG AFTER ACETYLCHOLINE ... chloroform (●) and ether (○) ... ercentage of the control. Lethal ... rations in human blood. Concentrations of chloroform above 0.16 cc per cent and of ether above 3.7 cc per cent cause contracture (represented by pyramidal columns of lines).

Wilmers, 7; Verzar, Bogel and Szanyi, 8; Bethe, Fraenkel and Wilmers, 9; Bethe, 10). The speed with which contracture develops increases with the concentration of chloroform. During the early stages of contracture the muscle still responds to acetylcholine by contraction. The presence of acetylcholine does not modify the concentrations of chloroform necessary to produce contracture.

Effect of ether on the response of striated frog muscle to acetylcholine. The effect of ether is qualitatively the same as that of chloroform, except that the smallest concentration of ether required for an effect is about 50 times higher, the peak of potentiation is about three times as great and the range of concentrations having a potentiating effect is much greater (table 2, fig. 1).

Larger concentrations of ether also cause a decrease in the response to acetylcholine. Muscles immersed in concentrated solutions of ether (more than half saturated) enter into contracture. The contracture is reversible in the early stages. The speed with which contracture develops increases with the increase of the concentration of ether.

Relation between the effect of chloroform, ether and physostigmine on the acetylcholine sensitivity of striated frog muscle. Experiments were also devised to study the mechanism of the anti-choline esterase activity of chloroform and ether to

TABLE 2

Effect of ether on muscle contraction from acetylcholine

CONCENTRATION OF ETHER	NUMBER OF EXPERIMENTS	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL	
		Mean	S.E.
cc. per cent			
0.050	11	107	±5.5
0.075	4	116	±8.4
0.100	12	145	±9.6
0.200	15	178	±15.7
0.500	29	328	±12.0
1.000	12	229	±14.2
1.500	15	91	±9.6
1.800	4	65	±10.3
2.000	6	0.3	±0.01
3.700	20	Contracture	

ascertain whether it differs from that of physostigmine. Muscles were immersed alternately for five minutes in solutions containing various concentrations of physostigmine ranging from 0.4 to 3.2 mgm. in 100 cc., for thirty seconds in acetylcholine, and for five minutes in Ringer's solution. The height of the acetylcholine contraction increased gradually to a certain degree, and eventually decreased. By increasing the concentrations of physostigmine, the peak of the potentiation is reached earlier. The potentiating effect of physostigmine tends to disappear with higher concentrations of the durg, but even with the highest concentrations used in these experiments the response of the muscle to acetylcholine was not diminished below the level prior to the addition of physostigmine (table 3). Chloroform increased the potentiating effect of physostigmine on the response of muscle to acetylcholine so that the two together may potentiate the effect of acetylcholine on the muscle to a greater degree than either alone (table 4). The potentiating effect of chloroform and ether occurs even after sixty minutes immersion in a 6 mgm. per cent physostigmine solution (table 5).

TABLE 3

Effect of physostigmine on muscle contraction from acetylcholine

A. Immersion for 5 minutes in physostigmine

CONCENTRATION OF PHYSOSTIGMINE	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL	
	Mean*	S.E.
mgm per 100 cc.		
0.001	113	±2.5
0.002	115	±1.9
0.005	115	±2.8
0.01	116	±2.3
0.05	119	±5.7
0.1	137	±10.2
0.4	169	±13.0
0.8	250	±15.4
1.0	275	±14.9
1.6	343	±13.3
3.2	488	±10.0
5.0	495	±14.8

* Each value represents the mean of 10 experiments.

B. Repeated immersion in physostigmine

CONCENTRATION OF PHYSOSTIGMINE	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL FOLLOWING SUCCESSIVE FIVE MINUTE IMMERSION PERIODS*							
mgm. per 100 cc								
0.4	169	244	270	244	227	202	174	148
0.8	250	332	302	274	246	213	199	162
1.6	343	370	339	244	175	135	118	106
3.2	488	512	381	194	150	118	110	101

* Each value represents the mean of 10 experiments. The S.E. of the mean for each value is less than ±10 per cent.

TABLE 4

Effect of chloroform on the acetylcholine contraction of muscle treated with a moderate amount of physostigmine

CONCENTRATION OF PHYSOSTIGMINE	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL* FOR VARIOUS CONCENTRATIONS OF CHLOROFORM (CC. PER CENT)							
	0.005	0.010	0.020	0.030	0.040	0.050	0.080	0.120
mgm. per 100 cc.								
0	157	164	121	96	86	67	37	35
0.4	178	336	409	349	304	254	145	70
0.8	305	439	403	282	207	156	47	13
1.6	460	443	342	191	120	55	35	9
3.2	731	490	305	139	51	33	13	0

* Each value represents the mean of from 10 to 46 experiments. The S.E. of the mean is in every case less than ±10 per cent.

If the muscle is first depressed by chloroform (0.050 cc. per cent) so that its response to acetylcholine is reduced about 40%, physostigmine still produces the potentiation of the effect of acetylcholine (table 6).

Combined effect of atropine, chloroform and ether. To investigate further the nature of the effect of chloroform and ether, experiments were carried out with atropine. Atropine in the concentration used (6 mgm. atropine sulfate per 100 cc. for 30 minutes) inhibits completely and in a reversible way the action of acetylcholine on the muscle without disturbing the action of direct stimulation of the muscle itself (potassium contraction). Chloroform in concentrations from 0 to 0.16 cc. per cent and ether in concentrations from 0 to 3.7 cc. per cent are not able to counteract this blocking effect of atropine (26 series of experiments), but the same amount of chloroform (0.14–0.16 cc. per cent) and ether (3.7 cc.

TABLE 5

Effect of chloroform and ether on the acetylcholine contraction of muscle after prolonged treatment with higher concentrations of physostigmine

DRUG	CC. PER CENT	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL			
		Muscle immersed for 1 hour in 6.4 mgm. per 100 cc. physostigmine solution		Muscle immersed in Ringer's solution	
		Mean*	S.E.	Mean†	S.E.
Chloroform	0	100		100	
	0.01	420	±10.9	164	±9.9
	0.08	20	±3.5	37	±8.2
Ether	0	100		100	
	0.05	150	±8.2	107	±5.5
	0.1	133	±10.4	145	±9.6
	0.5	46	±10.1	328	±12.0

* Each value represents the mean of 5 experiments.
† Each value represents the mean of from 5 to 31 experiments.

TABLE 6

Effect of physostigmine on the acetylcholine contraction of muscle treated with chloroform

CONCENTRATION OF PHYSOSTIGMINE	HEIGHT OF CONTRACTION IN PER CENT OF CONTROL			
	After immersion in 0.050 cc. per cent chloroform for 15 minutes		After immersion in Ringer's solution	
	Mean*	S.E.	Mean*	S.E.
mgm. per 100 cc.				
0	100		100	
0.4	133	±5.7	169	±13.0
0.8	285	±8.2	250	±15.4
1.6	290	±8.2	343	±13.3
3.2	136	±6.0	488	±10.0

* Each value represents the mean of 10 experiments.

per cent) is necessary to produce contracture of non-atropinized and atropinized muscle.

DISCUSSION. The experiments described above indicate that chloroform and ether modify the response of striated muscle to acetylcholine: (*1*) Low concentrations increase the response of the muscle to acetylcholine (chloroform from 0.0025 to 0.03 cc. per cent; ether from 0.05 to 1.5 cc. per cent). (*2*) Higher concentrations decrease the response of the muscle to acetylcholine (chloroform from 0.03 cc. per cent; ether from 1.5 cc. per cent). (*3*) Still higher concentrations cause contracture (chloroform from 0.16 cc. per cent; ether from 3.7 cc. per cent).

The behavior of the muscle indicates that the action of chloroform and ether is the result of at least two different processes, namely, potentiation and depression. Potentiation is more pronounced with lower concentrations, and depression gradually replaces potentiation with increased concentrations.

The potentiation is probably caused by two processes: inhibition of the activity of choline esterase resulting in a conservation of acetylcholine, and another not yet identified process. The inhibition of choline esterase is concluded from the following observations: (*1*) Chloroform and ether inhibit the choline esterase activity of muscles *in vitro* (1, 2). (*2*) Chloroform and ether even in high concentrations are not able to counteract the effect of atropine in inhibiting the effect of acetycholine.

That another process also operates in the potentiating effect of chloroform and ether may be assumed from the fact that the maximum response of muscle to acetylcholine may be greater with a combination of physostigmine and chloroform or ether, than with either alone.

The contracture caused by concentrated solutions of chloroform and ether is probably not the result of increased liberation or synthesis of acetylcholine because: (*1*) Acetylcholine does not decrease the concentrations of chloroform or ether necessary to produce a contracture; (*2*) atropine exerts no significant effect on the concentrations of chloroform and ether necessary to produce contracture.

An explanation for the difference in the effect of chloroform and ether in general anesthesia may be suggested from the results presented above: Figure 1 shows that stimulation of the parasympathetic nervous system is produced by much lower concentrations of chloroform than the lethal concentrations in the blood or tissues in man (40–60 mgm. per 100 cc. of blood, 11, 12, 13, 14), whereas in the case of ether, these concentrations are higher than the lethal concentrations in the blood or tissues of man (150 to 170 mgm. per 100 cc. of blood, 11, 15, 16).

SUMMARY

1. The effect of chloroform and ether on the response of the rectus abdominis muscle of frog to acetylcholine was investigated.

2. These drugs in low concentrations increase the response of the muscle to acetylcholine (chloroform from 0.0025 to 0.03 cc. per cent; ether from 0.5 to 1.5 cc. per cent).

3. Higher concentrations decrease the response of the muscle to acetylcholine (chloroform from 0.03 cc. per cent; ether from 1.5 cc. per cent).

4. Still higher concentrations cause contracture (chloroform from 0.16 cc. per cent; ether more than 3.7 cc. per cent).

5. Physostigmine potentiates the response of deeply chloroformed muscles to acetylcholine.

6. Chloroform and ether potentiate the response of fully eserinized muscles to acetylcholine.

7. Chloroform and ether even in high concentration are not able to counteract the effect of atropine in inhibiting the effect of acetylcholine.

8. Atropine does not modify chloroform and ether contracture.

9. These results indicate that chloroform and ether increase the excitability of the parasympathetic nervous system both by inhibiting the destruction of acetylcholine by choline esterase and by increasing the sensitivity of the effector organs to acetylcholine.

10. The contracture caused by chloroform and ether is probably the result of processes which do not involve acetylcholine.

11. The difference in the parasympathetic effects of chloroform and ether during anesthesia may be explained by the difference in concentrations required to stimulate the parasympathetic nervous system and the relative lethal concentrations in the tissues.

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SULFAMERIZINE[1] (2-SULFANILAMIDO-4-METHYLPYRIMIDINE)

I. A COMPARISON OF SULFAMERIZINE WITH SULFADIAZINE ON THE BASIS OF ABSORPTION, EXCRETION AND TOXICITY

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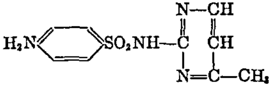
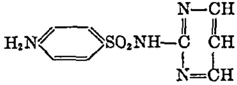
In an attempt to find a chemotherapeutic agent which possesses definite clinical advantages over sulfapyridine and sulfathiazole our attention was directed to various pyrimidine derivatives of sulfanilamide. Of these, 2-sulfanilamido-4-methylpyrimidine appeared in preliminary experiments to offer certain definite advantages over not only those sulfonamides which so far have been studied adequately, but also over other pyrimidine derivatives of sulfanilamide. The chemistry of this compound, which will be referred to as sulfamerizine, has been described in the publications of several groups: Roblin, *et al.* (1), Caldwell, *et al.* (2) and Sprague, *et al.* (3).



Sulfamerizine



Sulfadiazine

One of the related drugs which we have studied extensively, 2-sulfanilamidopyrimidine (sulfadiazine), has been described by Feinstone and his coworkers (4), who reported on its toxicity, absorption and chemotherapeutic activity. Subsequent clinical experience has demonstrated the effectiveness and relatively low toxicity of the compound, while the occurrence of certain side-effects has been notably less common than with sulfanilamide, sulfathiazole and sulfapyridine. Since the solubility of acetylsulfadiazine is greater than that of sulfadiazine, it was hoped that precipitation of drug in the urinary tract, with subsequent renal damage, might not develop with this compound. Experience has shown, however, that renal injury has occurred following the use of sulfadiazine (5–15), as has proved to be the case with several other sulfonamides. Following an

[1] Sulfamerizine: sul-fa-mĕr′-ĭ-zēne.

initial oral dose of drug the concentration of sulfadiazine in the blood usually rises rather slowly, in comparison with certain other sulfonamides; as a result many clinicians have made a practice of administering intravenously the initial dose of the drug as the sodium salt, when, as in pneumonia, it is desired to produce a high concentration in the body tissues without loss of valuable time.

On comparing sulfamerizine with sulfadiazine it is found that when the two compounds are given in identical oral dosage the sulfonamide concentration which is produced in the blood by the methyl compound is considerably greater; this difference in the blood level produced by equivalent doses becomes progressively more striking as the dosage of the two compounds is increased.

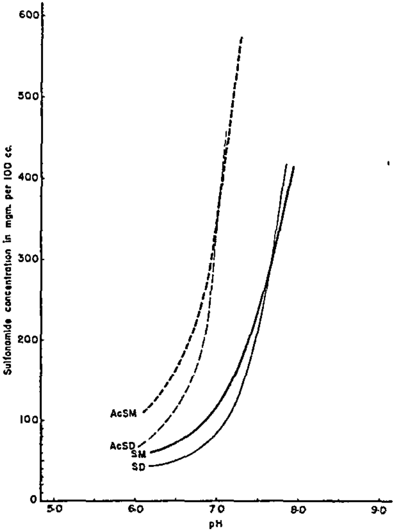
A given concentration in the blood, following oral dosage with sulfamerizine, is not only produced by a smaller dose, but also is more rapidly attained than with sulfadiazine, due apparently to the more rapid and more complete absorption of the methyl compound. Whether this increased rate of absorption will be of sufficient magnitude to obviate entirely any need for initial intravenous administration, will be evident only after additional clinical study.

Like acetylsulfadiazine, acetylsulfamerizine is more soluble than the unacetylated parent compound, and both sulfamerizine and its acetyl derivative are somewhat more soluble than the respective sulfadiazines (in urine at a pH of 7 or less) (see figure 1). This suggests that the danger of producing renal damage may be less with sulfamerizine than with sulfadiazine, and therefore markedly less than with sulfathiazole and sulfapyridine.

The clinical evidence (see review of Little (16)) indicates that sulfamethylthiazole and sulfanilyldimethylsulfanilamide exhibit a greater potentiality for causing neuropathological changes than do the commonly used sulfonamides. The presence of a methyl group *per se* offers no known chemical or pharmacological basis to justify a presumption that any sulfonamide containing a methyl group will be more productive of nerve injury than one lacking such a group; in fact, the compound which Bieter, *et al.* (17) found to be most damaging to chickens contains no methyl group (sulfaphenylthiazole). In our investigation particular attention has been given to the nervous tissues of dogs and monkeys, which received large doses of sulfamerizine or sulfadiazine three times daily for 30 day periods, and to the nervous tissues of chickens, a species remarkably sensitive to the nerve-damaging potentiality of sulfonamides (17, 18, 19). The data presented herein offer no evidence of nerve injury in dogs or monkeys; in chickens the nerve lesions which occur as a result of very high concentrations of sulfamerizine in the blood are of no greater severity than those resulting from lower blood levels of sulfadiazine. A search of the literature describing the extensive clinical studies of sulfadiazine has yielded only a single case (16) of nerve injury in man following its use. It would appear reasonable to suggest that nerve injury following the clinical use of sulfamerizine should be no more frequent than following sulfadiazine; the preliminary use of the drug in human beings has disclosed no evidence of neuropathological changes.

The practical significance of the observations made in animal species may be readily appreciated. In fact, preliminary studies in man show that sulfa-

merizine is efficacious in the treatment of those infections for which sulfadiazine has been found useful; that the drug is significantly more rapidly absorbed from the alimentary tract than is sulfadiazine; and that side-effects, as with sulfadiazine, are infrequent.



aid.

EXPERIMENTAL. Sulfamerizine (SM) (2-sulfanilamido-4-methylpyrimidine) has been studied extensively in several species (mice, rats, chickens, dogs, monkeys, men). The data have been obtained primarily from experiments in which the drug was given in large dosage for several days or weeks, in so-called chronic toxicity studies. The findings obtained from experiments conducted in rats and in dogs were considered of a preliminary character and were not controlled

routinely by determinations of sulfonamide concentration in the blood, urine or feces; the more detailed chronic studies carried out in monkeys, in which sulfamerizine was compared with sulfadiazine (SD), were supplemented by frequent determination of the sulfonamide concentration in the blood and in the excretions. In addition to the chronic toxicity studies, data were obtained from the repeated administration of small doses in monkeys, from single doses and repeated doses in men, and from single doses given for the determination of the acute toxicity of the drug in mice.

Acute toxicity. Mice. Sulfamerizine, suspended in 0.5% tragacanth and administered by stomach tube to white mice (Carworth CF_1), caused no toxic manifestations or fatalities, immediate or delayed, in the largest doses administered: 32 grams per kgm. Powell and Chen (20), using mice, found the LD_{50} of SD, suspended in acacia, to be greater than 34 grams per kgm.

The oral toxicity of sodium SM was determined at three different times using two strains of mice; the results, described below, are expressed in terms of SM and SD, rather than as the sodium salts. In the first test, white female mice (Carworth CF_1) were given varying doses of sodium SM in aqueous solution; the LD_{50} (Behrens method) was found to be 2.5 grams per kgm. Twenty-six months later a similar test was conducted using mixed male and female white mice (Swiss) in order to compare the oral toxicity of sodium SM and sodium SD. The LD_{50} of sodium SM was found to be aproximately 2.4 grams per kgm.; the LD_{50} of sodium SD was about 2.6 grams per kgm. Four months later the same compounds were again compared using male and female mice (Swiss) with the mice equally distributed as to sex over the entire range of dosage. The LD_{50} of sodium SM was determined to be approximately 2.6 grams per kgm., and that of sodium SD to be about 2.8 grams per kgm.; only those mice which died within 24 hours were considered in the construction of the toxicity curve. When *all* mice which died were used in the calculations, the LD_{50} of sodium SD was about 2.2 grams per kgm., while that of sodium SM (2.6 grams per kgm.) was unchanged.

It should be noted that following oral dosage of mice with sodium SM all deaths occur within 24 hours; the majority of animals die within 4 hours. On the other hand, the majority of mice intubated with sodium SD die between 24 and 48 hours, and some as late as 6 days, following the initial dosage. This is in accord with the findings of Powell and Chen (20) who noted late deaths following doses of sodium SD.

Gross and microscopic examination of the kidneys of the mice given sodium SM revealed vascular congestion, but no crystals could be found. The majority of the mice given sodium SD, which died 24 hours or more following dosage, had kidneys which revealed not only macroscopic and microscopic evidence of vascular congestion but also the presence of crystals in the tubules and pelves.

A study is now being made of the cause for delayed deaths in mice following the administration of large doses of SD.

Chronic toxicity. Rats. It has been found that SM produces no toxic effects when added to the diet of white rats to the extent of 3 to 5 %, for

periods of from 10 to 20 days, except those which might result from the precipitation of any sulfonamide in the urinary tract. All histopathological changes found were restricted to the kidneys and were consistent with those changes secondary to obstruction of the outflow of urine.

In a "paired-feeding" test, the growth rate of white rats[2] on a diet containing 0.5% SM was shown to be indistinguishable from that of animals receiving identical amounts of the same diet without drug. In this experiment one rat was given an adequate rat diet *ad libitum*; the food consumption of a second rat on the same diet plus 2% sulfapyridine was measured daily. On the following day the same amount of diet containing 0.5% SM was given to rat 3 (this is a dietary level which produces a sulfonamide concentration in the blood similar to that resulting from 2% sulfapyridine); rat 4 received an identical quantity of plain diet. Seven such groups were employed in the test, which lasted for a period of 30 days. The weight changes which resulted were averaged and are shown, with the standard errors, in the following:

DIET	INITIAL WEIGHT	CHANGE IN WEIGHT
	grams	*grams*
Plain diet—*ad libitum*	150 ± 5.9	+109 ± 4.9
Sulfapyridine—2.0%	151 ± 4.4	−7 ± 6.7
Sulfamerizine—0.5%	149 ± 4.5	+10 ± 4.3
Plain diet—restricted amount	148 ± 4.0	+3 ± 4.9

The rats on 2% sulfapyridine ate very much less food than those on the plain diet *ad libitum*. The weight changes produced in the rats on a severely, but identically, restricted caloric intake of plain diet suggest that, in the amounts fed, neither of the two drugs exerted any genuinely toxic effect on the animals. Although the appetite of the rats on sulfapyridine was greatly decreased there were no other deleterious influences of significance on these animals or on those given sulfamerizine, as measured by the ability of the rats to utilize for weight gain the food which was ingested. Examination of the tissues from these animals disclosed no histopathological changes.

Dogs. The animals were placed on a diet of dog pellets (Wayne) and raw meat. Sulfamerizine was administered in the form of tablets (0.5 gram and 0.125 gram) at 8 hour intervals. Vitamin capsules[3] were given once daily to reduce the chance of a deficiency developing in those vitamins of well-established significance. Three dogs were given 0.4 gram, three dogs 0.8 gram and three dogs 1.6 grams of sulfamerizine per kgm. daily. An additional group of three dogs, which served to control the experiment, remained in good health throughout the period, but at the end were found, as were the experimental animals, to be heavily parasitized with intestinal worms (tape worms, round worms, whip worms).

[2] Sunny Hill strain.

[3] Vitamin A, 10,000 U S.P. units, vitamin D, 1,000 U.S.P. units. Thiamine hydrochloride, 2 mgm.; riboflavin, 2 mgm ; niacin amide, 20 mgm

On the largest dose (1.6 grams per kgm. daily) one dog (#1) was killed in an emaciated condition after 32 days of dosage; #2 died after only 5 days of dosage, having refused essentially all food; #3 was sacrificed after 25 days, also having refused practically all food for several days. Histopathological study of these animals disclosed changes comparable to those found in starvation and anemia; although crystalline concretions were found in the pelves of the kidneys, it is important to emphasize that no significant changes in the kidney tubules or glomeruli were seen; the liver of #1 was normal for a starved animal, the livers of #2 and #3 showed areas of focal necrosis and degenerative changes; the bone marrow of all three animals showed some evidence of decreased hematopoietic activity.

On the medium dose (0.8 gram per kgm. daily) one dog (#4) was killed after 8 days, anorexia having been marked during the last few days of the period; #5 survived the entire period of 35 days; #6 expired on the 28th day a few minutes after vomiting the last dose of drug. The gross and microscopic findings were similar to those of the first series of animals, except that the bone marrow of #6 was hyperplastic.

On the lowest dose of sulfamerizine (0.4 gram per kgm. daily) the three dogs survived the entire 5 week period of dosage. Although these animals had concretions in the kidney pelves, none showed any other detectable gross or microscopic changes.

The well-known failure of the canine species to acetylate sulfonamides suggests that the kidney concretions found in these animals contained free sulfamerizine. The formation of acetylsulfamerizine, which occurs in many other species, may be considered advantageous in that it reduces the opportunity for the formation of concretions, since its solubility is greater than that of free sulfamerizine (figure 1). Although blood level determinations were not carried out regularly, subsequent experiments in monkeys indicate that the sulfonamide concentrations in the blood of these animals must have been very high. In those dogs which received 0.8 gram or more of SM per kgm. daily the appetite was usually suppressed to a marked degree, however, 0.4 gram per kgm. daily was well-tolerated for a period of 35 days, at the end of which time no pathological changes could be found except that of drug deposition in the kidney pelves. Because of the acetylation deficiency the dog is not a particularly favorable species for the study of this type of drug and further experiments were conducted in monkeys.

Monkeys (Macaca mulatta). During the study of the chronic toxicity of sulfamerizine 29 monkeys have been employed; these animals were subjected to autopsy at the end of a 30 day period and histopathological examination was made of all the significant tissues (see section on pathology). Of the 29 monkeys, 4 were controls, while 10 received sulfadiazine (SD) and 15 received sulfamerizine (SM).

1. In a preliminary study of toxicity in this species, SM was given in tragacanth suspension, by stomach tube, to each of two monkeys for a period of 30 days; a third animal was intubated concurrently with plain tragacanth (0.5%).

Intubations were made twice daily, at 8:00 A.M. and 5:00 P.M., according to the schedule employed by Feinstone, *et al.* (4); in all subsequent experiments, however, drugs were given regularly at uniform intervals of 8 hours each, for a period of 30 days.

The monkeys on the preliminary trial of SM were given 0.2 gram (monkey #1) and 0.4 gram (monkey #2) per kgm. daily (in two divided doses) for 30 days (series 1). No evidence of toxicity was seen at any time and neither gross nor microscopic evidence of pathological changes could be found in any tissue (see section on pathology—series 1). No drug was found in the kidneys.

2. A second group of 12 monkeys (series 2) was then placed on a more critically designed experiment. Five animals were given sulfamerizine in 0.5% tragacanth suspension by stomach tube every 8 hours for a period of 30 days; the dosages were 0.6 gram (monkey #20), 0.9 gram (#16), 1.2 grams (#25), 1.5 grams (#24), and 1.8 grams (#18) per kgm. per day, respectively. A similar group of 5 animals (#30, #19, #29, #28, and #26) was given sulfadiazine according to an identical schedule of dosage; concurrently, 2 control monkeys (#22 and #27) were intubated with a suspension of tragacanth. The volume of each injection was 20 cc. In addition, each of the animals was given 3 cc. of a yeast concentrate[4] once daily. After a few days on a diet of pellets of dog chow, which were not well eaten, the ration was changed to fresh fruits and vegetables, whole wheat bread and raw peanuts; the animals were fed twice daily, at the beginning and end of the laboratory day. The concentration of both "free" and "total" drug in the blood was determined at frequent intervals by the method of Bratton and Marshall (21, 22).[5] Analyses of the urine for the amount of "free" and "total" sulfonamides were made on those occasions when uncontaminated urine specimens could be obtained without catheterization.

Prior to the change from pellets of dog chow to a varied diet, weight loss was noted in the animals receiving SM, but not in those given SD. On the tenth day of dosage the monkey on the lowest dose of SM (0.6 gram per kgm. per day) died with tuberculous pneumonia; this early fatal result was probably contributed to by the drug but, as will appear from later experiments, it should not be taken as indicating that this dosage is one which will invariably cause fatal results (also, see subsequent section on pathology).

Seventeen days after the onset of dosage in the above group of monkeys, 3 animals were added to the series and placed on SM for a 30 day period. One control animal (#22), of the two which were started earlier, was sacrificed at the end of the initial 30 day period, the other (#27) was continued for a total

[4] The approximate composition of the yeast concentrate (3 cc.) was as follows: thiamin hydrochloride, 1.8 mgm., riboflavin, 0.6 mgm., niacin, 0.7 mgm., pyridoxine hydrochloride, 0.3 mgm.; pantothenic acid, 1.3 mgm.

[5] The use of p-toluenesulfonic acid (PTS), in place of trichloracetic acid, is strongly recommended in the determination of pyrimidine derivatives of sulfanilamide. A final concentration of 4% PTS (without added HCl) permits the hydrolysis of acetylated sulfamerizine within 1 hour at 100°C., and gives excellent recoveries under controlled conditions. The use of trichloracetic acid and hydrochloric acid yields erratic results, with "totals" often significantly lower than "frees."

of 47 days, and sacrificed at the same time as the 3 monkeys which were started later. These 3 animals were given SM according to the following dosage schedule: 0.2 gram (#21), 0.4 gram (#23), and 0.6 gram (#17) per kgm. per day (in 3 divided doses) for 30 days.

During the test period none of the animals which received SD died. In the group which received SM both deaths and survivals occurred: those monkeys which received 0.2 gram (#21), 0.4 gram (#23), 0.6 gram (#17) and 1.2 grams (#25) per kgm. per day survived the entire period; in the animal which received 1.2 grams per kgm. per day (#25) tubular degeneration and dilatation in the kidney and hyperplasia of the bone marrow were found. Those monkeys which received the other doses of SM died at variable time intervals; the death of the animal on 0.6 gram per kgm. per day (#20) has been commented on previously; on 0.9 gram (#16), 1.5 grams (#24), and 1.8 grams (#18) per kgm. per day death occurred on the 28th, 22nd and 12th day of dosage, respectively. In each case deaths were accompanied by extensive tubular degeneration and dilatation in the kidney, but lesions in the glomeruli were not found; changes in the bone marrow were also noted (see section on pathology). Examination of the monkeys, #21, #23, #17, which were given dosages of 0.6 gram, or less, of SM per kgm. disclosed no signs of either gross or microscopic injury due to the drug and no drug was found in the kidneys.

If attention were not given to the concentrations of sulfonamide produced in the blood by these two drugs, the findings presented would appear to indicate that sulfadiazine is much less toxic than sulfamerizine. We agree with the statement of Feinstone *et al.*, in their study of SD (4), that drug concentration in the blood is a better basis for comparison between two drugs than is drug intake. In table 1 the data show that in this series of monkeys oral dosages of SD of from 1.2 to 1.8 grams per kgm. per day were required to produce a blood level of "free" sulfonamide in the same range as that produced by only 0.2 to 0.4 gram per kgm. per day of SM.

In this series of monkeys no manifestations of toxicity or tissue damage were seen in the animals in which the average 8 hour blood concentration did not exceed 27.4 mgm. % (free) SM. Tissue injury, usually fatal within a 30 day period, apparently occurred in those animals in which the average 8 hour blood concentration was 32.3 mgm. % or greater. However, monkey #20, with a blood level of 32.3 mgm. %, died suddenly with tuberculous pneumonia; certainly the average 8 hour sulfonamide concentration of 38.9 mgm. % shown by monkey #25 may be considered as definitely harmful to the renal tissue of the monkey (see section on pathology).

Dosages of SD as high as 1.8 grams per kgm. per day had no apparent deleterious effect, probably because the highest average 8 hour blood level produced by this drug in this series of monkeys was only 12.5 mgm. % (free) SD. The apparently greater toxicity of SM is due, it would seem, to the fact that the drug concentration in the blood produced by SM is very much greater than that which is produced by an equivalent dosage of SD.

3. A third group of monkeys (series 3) was given sulfamerizine and sulfa-

diazine in dosages so calculated that similar ranges of sulfonamide concentrations in the blood were expected. Three monkeys (#31, #33 and #35) were intubated every 8 hours with 20 cc. of tragacanth suspensions of SM in doses totaling 0.5, 0.6, and 0.7 gram per kgm. per day, respectively. Three monkeys (#32, #34, and #37) were given SD by the same technic with dosages of 1.8, 2.1, and 2.4 grams per kgm. per day, respectively. A seventh animal (#36), which served as a control, was given concurrent intubations of tragacanth suspension. In this experiment pellets of Purina dog chow and oranges furnished the entire source of calories. Adequate intake of the vitamin B complex was assured by the daily administration of 5 cc. of a yeast concentrate supplemented with synthetic members of the B-complex.[6]

TABLE 1

Average concentration of sulfonamides (in mgm. per 100 cc.) in the blood of monkeys following the oral administration of sulfamerizine (SM) and of sulfadiazine (SD)

The dose of each drug was divided into three portions and given every eight hours.

DOSE/24 HOURS	MONKEY NUMBER	AVERAGE CONCENTRATION OF SULFADIAZINE IN BLOOD		MONKEY NUMBER	AVERAGE CONCENTRATION OF SULFAMERIZINE IN BLOOD		REMARKS CONCERNING SULFAMERIZINE MONKEYS
		2 hours	8 hours		2 hours	8 hours	
grams/kgm.							
0.2				21	18.4	10.3	No illness; normal tissues.
0.4				23	22.6	13.2	No illness; normal tissues.
0.6	30	12.5	5.0	17	30.6	27.4	No illness; normal tissues.
0.6				20	31.8	32.3	Died of tuberculous pneumonia 10th day; renal damage, crystals.
0.9	19	16.2	6.7	16	55.6	44.7	Died 28th day; crystals, renal damage.
1.2	29	18.7	12.5	25	54.7	38.9	Sacrificed on 31st day; renal damage.
1.5	28	21.4	9.6	24	73.8	67.1	Died 22nd day; crystals, renal damage.
1.8	26	23.4	11.5	18	67.1	62.4	Died 12th day; crystals, renal damage.

After about 10 days it became evident that water bottles attached to the cages failed adequately to supply the animals. As a result of restricted water consumption and decreased elimination of urine by the kidney, the drugs were retained in the body and the sulfonamide concentrations in the blood rose to very high levels. It should be noted, however, (table 2) that the monkey (#35) on the highest dose of SM (0.7 gram per kgm. per day) did not accumulate as large an amount of sulfonamide as the other two animals on SM; following the restoration of a fully adequate water supply the sulfonamide concentration in the blood of this animal fell to a plateau similar to that attained within 24 to 48 hours following the initiation of dosage. Two of the animals on SD (#34 and #37) showed a similar drop in sulfonamide blood level after the restoration

[6] The approximate composition of the supplemented yeast concentrate (5 cc.) was as follows: thiamin hydrochloride, 1.8 mgm.; riboflavin, 3.6 mgm.; niacin, 20.7 mgm.; pyridoxine hydrochloride, 2.3 mgm., pantothenic acid, 21.3 mgm.

of water balance, but the monkey (#32) on the smallest dose of SD (1.8 grams per kgm. per day) never regained adequate kidney function. During the last few days of intubation this monkey (#32) gradually passed into coma and, on the 29th day of dosage, became moribund; at the time of sacrifice the body temperature had fallen to only 27°C. In table 2 are presented the concentrations of SM and of SD in the blood throughout the experiment.

There was a noticeable increase in the percentage of acetylated drug in the blood during the period of marked sulfonamide retention. Under normal circumstances the low concentration of the acetylated form of SD in the blood depends upon the rapid urinary excretion of this derivative. When renal function is inadequate, as in the monkeys just described, the amounts of both the free and the acetylated sulfonamide in the blood rise to very high levels. In those animals in which proper kidney function was regained (#35 on SM; #34 and #37 on SD) the amount of conjugated drug in the blood fell steadily to an insignificant level. Monkey #32, on 1.8 grams of SD per kgm. per day, never regained adequate kidney function and was sacrificed on the 30th day in a moribund state with an extremely high blood level of both free and conjugated drug (142.5 mgm. % free and 97.4 mgm. % conjugated SD). Monkeys #31 and #33, on 0.5 and 0.6 gram of SM per kgm. per day, respectively, died at a time when it was not possible to estimate terminal blood levels; however, #31 had, on the day during which death occurred, a blood level of 43.3 mgm. % free and 62.7 mgm. % conjugated SM, while #33 had, on the day prior to death, a blood level of 78.6 mgm. % free and 57.4 mgm. % conjugated SM.

These data emphasize the importance of an ample intake of water to insure adequate urine volume and thus to minimize the danger of precipitation of sulfadiazine or sulfamerizine in the urinary tract. This is particularly true, of course, when sulfonamide concentrations in the blood are maintained at levels higher than are customarily employed in clinical practice. In all these animals, with the exception of monkey #35, the concentration of drug in the blood rose to such high levels that, with the limited output of urine, precipitation of drug in the kidney tubules was almost inevitable; the entire nephron whose tubule was so affected probably became functionless. Presumably a sufficient number of nephrons remained intact, in those monkeys which recovered when water balance was restored, to enable the animal to survive the kidney deficiency, and, with the exception of monkey #32, gradually to eliminate the deposited drug. Furthermore, it is interesting to note that recovery occurred in those monkeys which were given the highest dosages of each drug.

4. A final group of monkeys (series 4) was begun during the course of the experiment last described. The purpose of this test was, as in series 3, to produce closely similar, high blood levels of SM and of SD, which would enable certain comparative studies of excretion to be made; in addition, it was desired to determine, under conditions of unlimited water intake, the concentration of sulfadiazine in the blood and other tissues necessary to cause death within a period of less than 30 days. For the first purpose one animal (#42) was placed on 0.5 gram and another (#44) on 0.6 gram of SM per kgm. per day (in 3 divided

TABLE 2

Concentration of sulfonamides (in mgm. per 100 cc.) in the blood of monkeys following the oral administration of sulfamerizine (SM) and of sulfadiazine (SD)

The dose of each drug was divided into three portions and given every eight hours. During the first 10 days of the experimental period these animals received inadequate amounts of water.

TIME	0.5 GRAM PER KGM. SM, #31		0.6 GRAM PER KGM. SM, #33		0.7 GRAM PER KGM. SM, #35		1.8 GRAMS PER KGM. SD, #32		2.1 GRAMS PER KGM. SD, #34		2.4 GRAMS PER KGM. SD, #37	
	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
1 hr.	15.4	1.0	14.6	0.8	13.1	0.9	5.8	0.7	4.8	0.7	10.3	0.7
2 hrs.	15.2	0.8	23.7	1.1	18.1	1.9	9.8	0.7	11.8	0.5	18.9	0.5
4 hrs.	15.5	0.8	24.6	1.9	15.3	3.7	11.3	0.5	17.1	1.3	19.9	4.5
8 hrs.	14.9	1.1	15.1	3.3	10.6	1.5	8.0	0.7	13.7	1.3	14.8	0.7
24 hrs.	19.7	2.7	24.6	1.4	24.8	2.8	23.9	0.7	20.5	0.5	29.4	1.1
48 hrs.	28.3	5.9	22.2	3.6	26.6	1.2	16.3	0.6	18.3	0.6	40.1	3.5
74 hrs.	45.6	43.1	53.7	14.5	27.6	0.9	48.0	5.6	47.2	0.9	57.0	6.1
76 hrs.	49.3	31.2	47.6	27.2	30.4	0.8	51.9	6.9	52.7	1.6	60.8	3.4
80 hrs.	52.8	42.2	49.1	36.9	19.1	2.8	48.0	9.1	38.4	3.2	53.7	6.2
8th day—2 hrs.	52.2	52.8	64.5	51.5	39.0	3.0	64.6	11.9	65.6	24.7	66.5	21.7
4 hrs.	53.7	52.3	65.5	52.5	40.1	15.1	65.9	12.8	69.5	28.8	71.2	23.0
8 hrs.	48.6	59.4	47.6	49.6	27.0	9.4	56.6	17.0	56.4	30.8	63.8	26.4
11th day—2 hrs.	62.7	58.3	72.1	50.9	49.8	3.3	69.7	9.2	64.5	7.9	63.3	2.5
4 hrs.	62.3	54.7	65.6	20.9	47.8	2.0	68.6	5.7	65.4	9.0	65.1	8.0
8 hrs.	58.9	31.7	59.6	50.4	35.6	2.1	55.9	11.8	59.9	9.9	64.7	25.5
15th day—2 hrs.	57.0	64.0	96.7	53.3	50.7	3.9	58.4	28.6			64.1	5.0
4 hrs.	53.7	65.3	89.4	92.6	45.2	3 2	52.3	43.8	37.1	4.4	61.0	13.4
8 hrs.	43.3	62.7	78.6	57.4	31.5	3.2	60.6	19.8	27.8	5.9	48.4	7.1
18th day—2 hrs.	Died—15th day		Died—16th day		38.4	5.3	74.0	17.5	27.7	2.1	54.0	4.7
4 hrs.					40.0	3.7	74.0		27.0	2.6	56.2	3.3
8 hrs.					28.4		82.9	7.3	20.7	9.1	58.1	
22nd day—2 hrs.					36.7	18.7	73.0	20.5	28.2	1.7	44.4	8.1
4 hrs.					34.0	2.5	72.1	18.0	32.0	9.6	48.1	4.4
8 hrs.					26.1	1.8	85.7	5.7	29.6	0.3	42.7	13.9
25th day—2 hrs.					37.3		95.7		19.6		39.2	
4 hrs.					34.8		97.1		19.0		40.5	
8 hrs.					25.4	0.6	97.1	32.9	11.2	0.8	34.5	
29th day—2 hrs.					30.6	1.2	65.2	25.6			34.5	0.5
4 hrs.					30.6	0.9	68.3	28.0			33.9	0.3
8 hrs.					22.8	0.6	64.4	38.3			34.8	0.2
30th day—0 hr.									10.1			
2 hrs.									21.8	0.8		
4 hrs.									19.3			
8 hrs.							74.1	38.9	11.0	0.6		
14 hrs.							142.5	97.4				
							(killed)					

doses); a third monkey (#43) was given 2.4 grams of SD per kgm. per day. For the second purpose a fourth animal (#45) was given 3.0 grams of SD per kgm. per day. The volume of the sulfonamide suspensions given was propor-

tional to the body weight of each monkey; 5 cc. of a yeast concentrate supplemented with synthetic members of the vitamin B complex[6] were given daily.

After about 10 days it was evident that the concentration of SD in the blood of monkey #43 (2.4 grams) was higher than was desired; accordingly the animal was taken off drug for a period of 48 hours (no toxic symptoms had been evident) and then given, for the remainder of the period, 1.8 grams of SD per kgm. daily in 3 divided doses. At the same time (10th day) the dosage of #44 was increased from 0.6 gram to 0.9 gram of SM per kgm. per day. It was hoped that in this manner the blood levels of monkeys #43 and #44 might eventually approximate each other.

During the remainder of the period of dosage the concentration of sulfonamides in the blood of the two monkeys on SM (0.5 and 0.9 gram per kgm. per day, respectively) and in the animal on 1.8 grams per kgm. per day of SD, were not remarkably different (table 3). It will be noted that the level of conjugated drug in the blood of these three animals remained low; in the monkey on 3.0 grams of SD per kgm. per day, however, retention of the acetylated form was at times considerable. The pathological findings in these four animals show that only in the animal (#45) in which marked retention of acetylsulfadiazine occurred was kidney damage evident; of course, the concentration of free sulfonamide in the blood of this animal reached a very much higher level than was the case in any of the other three monkeys.

During the second week, when the blood level was very high, the monkey (#45) on 3.0 grams of SD per kgm. became acutely ill and was expected to die. However, spontaneous, striking improvement occurred, and the blood level fell lthough there was no change in the manner of dosing, handling or feeding the nimal. The improvement in condition was doubtless related to the decrease the free and conjugated sulfonamide content of the tissues; while the reason or this change is not clearly evident, conceivably it may have been due to an lteration in the ability of the kidney tubules to reabsorb sulfadiazine and its cetylated derivative. Microscopic study disclosed extensive tubular damage n the kidneys of this animal.

The monkeys on SM (0.5 and 0.9 gram per kgm.) and the monkey on the ower dosage of SD (1.8 grams per kgm.) showed no ill effects from the drugs and aintained sulfonamide concentrations in the blood of a similar order of magitude (table 3). Gross and microscopic examination of the tissues of these nimals disclosed no lesions which could in any way be attributed to the drug reatment.

Feinstone, *et al.* (4), from their data on SD, concluded that "monkeys remained ormal after maintaining blood concentrations up to 20 mgm. % of free sulfadiazine and 2 mgm. % of conjugated sulfadiazine in the blood stream." The conclusion of these workers was drawn from blood level data obtained 16 hours following one-half the daily dose. The lowest concentrations of sulfonamides which occurred in our animals were those found immediately preceding dosage, at the regular interval of 8 hours. Under such conditions, average 8-hour sulfamerizine and sulfadiazine concentrations in the blood of at least 25 mgm. %

(and 2 to 3 mgm. % of the conjugated form) appeared to produce no evidence of tissue damage. Of course, as has been emphasized previously, the output of

TABLE 3

Concentration of sulfonamides (in mgm. per 100 cc.) in the blood of monkeys following the oral administration of sulfamerizine (SM) and of sulfadiazine (SD)

The dose of each drug was divided into three portions and given every 8 hours.

TIME	0.5 GRAM PER KGM. SM, #42		0.6 GRAM PER KGM. SM, #44		2.4 GRAMS PER KGM. SD, #43		3.0 GRAMS PER KGM. SD, #45	
	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
1 hr.	14.7		14.1	0.4	3.2	0.7	6.9	0.3
2 hrs.	18.4		19.9		9.6		11.4	
4 hrs.	18.9		16.2		12.7		11.5	
8 hrs.	12.4		10.4	1.1	9.2		8.1	
24 hrs.	16.9	0.9	13.5	1.4	15.6	0.8	10.8	0.9
48 hrs.	19.5	1.0	20.5	2.0	28.0	1.7	20.8	0.5
7th day—2 hrs.	34.1	2.2	30.8	2.7	63.1	19.5	51.9	13.6
4 hrs.	33.2	2.6	27.7	2.9	66.6	17.4	54.9	16.4
8 hrs.	29.3	3.2	21.9	2.0	64.4	11.7	59.9	20.4
10th day—2 hrs.	39.4	1.9	31.2	1.1	67.2	6.4	74.7	32.3
4 hrs.	29.8	2.2	24.4	1.4	58.0	6.5	75.3	30.7
8 hrs.	24.1	0.8	17.8	1.7	51.5	8.5	69.3	31.7
Dosage changed for Monkeys #44 and #43			On tenth day dosage increased to 0.9 gram per kgm. of SM		On tenth day drug withdrawn; two days later dosage renewed with 1.8 grams per kgm. of SD			
14th day—2 hrs.	24.9	1.6	36.8	2.9	30.0		47.5	29.9
4 hrs.	28.3	0.3	32.8	2.3	31.1		45.1	26.1
8 hrs.	22.1		25.9	0 9	27.8	0.5	43.6	24.7
17th day—2 hrs.	31.5	0.4	36.3	0.3	29.3	0.2	37.1	6.7
4 hrs.	29.3	0.2	34.5	0.9	24.9	1.4	36.8	4.9
8 hrs.	22.4		27.7		21.4		37.0	3.2
21st day—2 hrs.	34.2	0.3	31 6	1.4	21.6	0.6	58.5	0.3
4 hrs.	28.7	1.2	29.6	0 3	23.0		55.2	4.4
8 hrs.	21.8	0.5	22.6	0.2	18.6	0.7	48.4	3.5
24th day—2 hrs.	30.8	1.7	30.5	0.7	23.0	0.4	59.3	8.0
4 hrs.	25.5	1.0	26.0	0.3	21.2	0.9	65.0	14.3
8 hrs.	20.1		17.0	0.8	19.0	1.5	60.0	7.7
28th day—2 hrs.	27.8		36.6	0.8	24.0		41.0	0.6
4 hrs.	22.5	1.0	29.4	0.3	22.1	0.8	41.7	0.3
8 hrs.	15.7	0.5	20.2		13.8	0.5	35.4	1.7

urine must be sufficient to minimize any danger of precipitation of either drug in the urinary tract.

The dosage of sulfamerizine required to produce high concentrations in the blood is usually from about one-fourth to one-half that which is required when sulfadiazine is used (tables 1, 2 and 3). The data of tables 2 and 3 also show

that the lower dosages of sulfamerizine produce significant concentrations in the blood very much more rapidly than is the case with the high dosages of sulfadiazine. Other data will be presented which also demonstrate the significantly more rapid rate of absorption of sulfamerizine from the gastrointestinal tract.

The urinary excretion of free and acetylated SM and SD by the monkeys (tables 4 and 5) yields information of considerable interest with relation to the

TABLE 4

Concentration of sulfonamides in the urine of monkeys receiving sulfamerizine (SM) or sulfadiazine (SD) orally in the dosage indicated (divided into 3 equal portions and given at 8 hour intervals)

These monkeys received inadequate amounts of water during the first 10 days of the test; animals #35, 34 and 37 gradually recovered from the resultant sulfonamide retention.

TIME	MONKEY #31, 0.5 GRAM SM		MONKEY #33, 0.6 GRAM SM		MONKEY #35, 0.7 GRAM SM		MONKEY #32, 1.8 GRAM SD		MONKEY #34, 2.1 GRAM SD		MONKEY #37, 2.4 GRAM SD	
	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
	mgm. per cent	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
4th day—2 hrs.					316	248						
8th day—2 hrs.					216	181	249	257				
4 hrs.	170	75					259	55				
10th day—8 hrs.	210	407			113	105						
12th day—1 hr.	312	323										
15th day—1 hr.	130	423										
16th day—post mortem			129	237								
18th day—8 hrs.					202	118						
26th day—2 hrs.					833	247	221	156	90	24	295	21
29th day—2 hrs.					445	323	206	140			347	43
4 hrs.					663	323	221	141			327	106
8 hrs.					514	300	197	129			229	36
30th day—2 hrs.									444	67		
4 hrs.									283	45		
8 hrs.							186	42	154	146		
31st day—6 hrs.							316	115				
12 hrs.									110	5		
15 hrs.											141	16
17 hrs.					207	134						

high proportion of conjugated sulfonamide excreted. Although the restricted water intake of the first 10 days in the monkeys described in tables 2 and 4, complicates the interpretation of the urinary sulfonamide concentrations in these animals, it is of interest to correlate the urinary with the blood sulfonamide concentrations in the last group of monkeys, particularly #42 and #43 (tables 3 and 5).

It will be noted that the degree of acetylation of these pyrimidine derivatives of sulfanilamide fluctuates considerably, as indicated by the proportion and

amount of the conjugated compound excreted in the urine. The concentration of the acetylated derivative in the blood usually remains low, apparently due to rapid elimination by the kidneys and not because either sulfadiazine or sulfamerizine is acetylated with difficulty. The high proportion of acetylated sulfadiazine which is excreted in the urine is evident from the data obtained by Zozaya (23) in experiments with rats, and from the data of Sadusk and Tredway (24) obtained in man. In both species it was found that the percentage of conjugated drug found in the urine following sulfadiazine administration tends

TABLE 5

Concentration of sulfonamides in the urine of monkeys receiving sulfamerizine (SM) or sulfadiazine (SD) orally in the dosage indicated (divided into 3 equal portions and given at 8 hour intervals

TIME	MONKEY #42, 0 5 GRAM SM		MONKEY #44, 0.6 GRAM SM		MONKEY #43, 2.4 GRAM SD		MONKEY #45, 3.0 GRAM SD	
	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
	mgm. per cent	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm per cent*
7th day—2 hrs.	370	352			133	135	511	341
4 hrs.	406	114			143	156	408	117
8 hrs.	85	120			125	159	389	358
10th day—2 hrs.			Dosage increased to 0 9 gram per kgm.		175	139		
11th day—8 hrs.					128	77		
12th day					Dosage changed to 1.8 gram per kgm. after 48 hrs. without dosage			
14th day	218	28			284	93	104	146
17th day—2 hrs.	288	162					88	86
4 hrs.			641	633	169	46		
21st day—2 hrs.					188	33	223	92
28th day—4 hrs.	81	44					189	92

to be somewhat higher than is the case with sulfathiazole. Fortunately the solubility of acetylsulfadiazine in urine at various pH levels is greater than that of acetylsulfathiazole. As is shown in figure 1 the solubility of acetylsulfamerizine is somewhat greater than that of acetylsulfadiazine in urine at a pH of 7 or less.

PATHOLOGICAL FINDINGS IN MONKEYS. In this section, the data are presented in the following order. number of monkey, drug; dose in grams per kgm. per day (in three divided doses) for 30 days; average minimal blood concentration (i.e., average level immediately prior to dosage at 8 hour intervals), state of animal during test; weight change; concentration of hemoglobin (Hb) in grams per 100 cc.; number of erythrocytes (r.b.c.) in millions per cmm. The gross and microscopic findings at autopsy are given, with attention directed routinely to kidneys, liver, bone marrow, sciatic nerve, spinal cord, bladder, ureter, lung,

heart, spleen, stomach, duodenum, pancreas, gall bladder, ileum, colon, adrenal, lymph node, and—in the last group of monkeys—thyroid. Weekly determinations were made, at the beginning of the experiment and during its course, of the erythrocyte count, total and differential leucocyte counts, and of the concentration of hemoglobin and of plasma proteins; in some cases the findings are presented only when significant deviations from the normal range were encountered (see Welch, Mattis and Latven (25)).

Series 1.

Monkey #1; SM; 0.2 gram; ———; survived and well; weight—3.5 to 3.3 kgm.: all tissues normal grossly and microscopically.

Monkey #2; SM; 0.4 gram; ———; survived and well; weight—3.6 to 3.4 kgm.: all tissues normal grossly and microscopically.

Monkey #3; Control; survived and well; weight—3.4 to 3.2 kgm.: all tissues normal grossly and microscopically.

Series 2.

Monkey #30; SD; 0.6 gram; average, 5.0 mgm. % free SD; survived and well; weight—4.2 to 4.8 kgm.; Hb, 16.5 grams % at beginning and end of 30 day period; r.b.c., 6.8 to 5.1 millions. All tissues normal grossly and microscopically.

Monkey #19; SD; 0.9 gram; average, 6.7 mgm. % free SD; survived and well; weight—3.9 to 4.6 kgm.; Hb, 14.4 rose to 18.0 grams %; r.b.c., at end of period 5.0 millions. Pulmonary tuberculosis; several renal tubules contained red blood cells; otherwise the tissues were normal grossly and microscopically.

Monkey #29; SD; 1.2 grams; average, 12.5 mgm. % free SD; survived and well; weight—3.8 to 4.4 kgm.; Hb, 14.4 to 13.5 grams %; r.b.c., 5.95 to 5.1 millions. Pulmonary tuberculosis; kidney—slight interstitial inflammatory infiltration; otherwise the tissues were normal grossly and microscopically.

Monkey #28; SD; 1.5 grams; average, 9.6 mgm. % free SD; survived and well; weight—.3 to 4.0 kgm.; Hb, 13.8 to 14.5 grams %; r.b.c., 6.6 to 5.1 millions. Early pulmonary uberculosis; kidney—ulceration of renal pyramid, inflammation of pelvis, hyperplasia of ubular epithelium; otherwise the tissues were normal grossly and microscopically.

Monkey #26; SD; 1.8 grams; average, 11.5 mgm. % free SD; survived and well; weight—.3 to 4.0 kgm.; Hb, 13.8 to 14.5 grams %; r.b.c., 6.6 to 5.1 millions. Early pulmonary uberculosis; otherwise the tissues were normal grossly and microscopically.

Monkey #21; SM; 0.2 gram, average, 10.3 mgm. % free SM; survived and well; weight—.8 to 5.1 kgm.; Hb, 12.9 to 11.2 grams %; r.b.c., 6.1 to 5.6 millions. Early pulmonary uberculosis; otherwise the tissues were normal grossly and microscopically.

Monkey #23; SM; 0.4 gram; average, 13.2 mgm. % free SM; survived and well; weight—.6 to 5.3 kgm.; Hb, 15.2 to 12.2 grams %; r.b.c., 6.2 to 5.8 millions. Parasitic infestation of ungs and colon; kidney—slight intercapillary hyalinization in glomeruli; otherwise the issues were normal grossly and microscopically.

Monkey #17; SM; 0.6 gram; average, 27.4 mgm. % free SM; survived and well; weight—.2 to 4.3 kgm.; Hb, 17.9 to 15.2 grams %; r.b.c., 6.7 to 5.7 millions. Parasitic infestaion of lungs; otherwise the tissues were normal grossly and microscopically.

Monkey #20; SM; 0.6 gram; average, 32.3 mgm. % free SM; toxic in appearance on 8th lay to death on 10th day with tuberculous pneumonia; weight—no change; Hb, 15.2 to 15.2 rams %; r.b.c., 7.5 to 7.1 millions. Cut surface of kidneys pale with concretions in pelves; oncretion in one ureter with ulceration at site; bladder—normal; lungs—caseation necrois, atelectasis; liver—small area of necrosis (tuberculous); lymph nodes—hypertrophied nd sometimes caseous. Microscopic findings: extensive pulmonary tuberculosis; paraitic lesions in colon; kidney—extensive damage, particularly tubular degeneration and lilatation, cellular casts, outlines of crystals in the tubules, glomeruli—essentially normal, hrombi in small renal veins; bone marrow—hyperplasia.

Monkey #16; SM; 0.9 gram; average 44.7 mgm. % free SM; occasionally regurgitated mmediately following intubation (reinjected); died suddenly on 28th day without previous vidence of illness; weight—4.3 to 5.6 kgm.; Hb, 13.4 to 9.0 grams %; r.b.c., 6.5 to 3.0 mil-

lions. Caseous tubercle in upper lobe of left lung; kidneys—cut surfaces showed crystalline deposits throughout, no concretions in pelves or in ureters; adrenals—hemorrhagic cortices, nodule in one medulla. Microscopic findings: lungs—early tuberculosis; kidneys—extensive damage, particularly tubular degeneration and dilatation, cellular casts, outlines of crystals in the tubules, glomeruli—essentially normal, thrombi in small renal veins; bone marrow—hyperplasia; adenoma of adrenal medulla.

Monkey #25; SM; 1.2 grams; average, 38.9 mgm. % free SM; survived and apparently well; 3 8 to 3.5 kgm.; Hb, 15.8 to 9.8 grams %; r.b.c., 6.5 to 2.9 millions. Lungs—few scat- [illegible] tion, cellular casts, outlines of crystals in the tubules, glomeruli—essentially normal, thrombi in small renal veins; bone marrow—hyperplasia.

Monkey #24; SM; 1.5 grams; average, 67.1 mgm. % free SM; toxic in appearance on 8th day to death on 22nd day; vomiting on one occasion only; apathetic; bloody urine noted on 8th day, but not thereafter; edema of eyelids and pelvic region noted toward end of life; weight—3 6 to 3.3 kgm.; Hb, 14.3 to 9.0 grams %; r.b.c., 6.3 to 3.4 millions in 15 days. Lungs—a few small discrete, caseous nodules; kidneys—pale in color, hemorrhagic areas on external surface; left kidney swollen and tense, subcapsular surface showed areas of opacity, with some petechiae, cut surface of kidney showed small hemorrhages, yellow opaque patches and microcrystalline deposits, pelvis filled with granular concretions, left ureter blocked by and dilated above a concretion; right kidney less swollen and less tense, general picture similar to that of left kidney, concretions in pelvis and in ureter, but no blockage. Microscopic findings: kidneys—extensive damage, particularly tubular degeneration and dilatation, cellular casts, outlines of crystals in the tubules, glomeruli—essentially normal, thrombi in small renal veins; liver—cells contain fat droplets; bone marrow—hyperplasia; myocardium—atrophy and degenerative lesions.

Monkey #18; SM; 1.8 grams; average, 62.4 mgm. % free SM; occasional regurgitation following intubation (reinjected); died on the 12th day of dosage; weight—3.2 to 3.0 kgm.; Hb, 15.8 to 15.0 grams %; r.b.c., 7.2 to 5.9 millions. Lungs—left lower lobe, small discrete yellowish-nodules throughout parenchyma, cut surfaces of nodules revealed cavitation and caseous material, other lobes appeared normal; heart—generalized subendocardial hemorrhage throughout left ventricle, thickened valvular edges; kidneys—cut surfaces demonstrated crystalline deposits throughout, concretions in pelves and in ureters but no blockage. Microscopic findings: kidneys—extensive damage, particularly tubular degeneration and dilatation, cellular casts, outlines of crystals in the tubules, glomeruli—essentially normal, thrombi in small renal veins; bone marrow—aplasia; lungs—tuberculosis.

Monkey #22; Control; survived and well, weight—4.1 to 4 9 kgm.; Hb, 12.2 to 16.8 grams %; r.b.c., 5.7 to 5.6 millions. Lungs—many small nodules, some calcified, some caseous, scattered throughout parenchyma. Microscopic findings: lungs—tuberculosis and lymphoid hyperplasia.

Monkey #27; Control; survived and well; weight—3.0 to 4.4 kgm. during a 47 day period; Hb, 14.0 to 12.8 grams %; r.b.c., 6.3 to 5.8 millions. Lungs—many small nodules, some calcified, some caseous, scattered throughout parenchyma. Microscopic findings· lungs—tuberculosis and lymphoid hyperplasia.

Series 3.

Reference should be made to table 1 for a correct appreciation of the blood level data, since the animals of this series all temporarily suffered from dehydration, correction of which was begun on the 11th day, as is explained elsewhere. The average blood levels given below (taken just prior to dosage) often do not indicate the high concentrations of sulfonamides attained in the blood during the dehydration period, or the recovery which occurred in some cases (#34, #37, #35)

Monkey #32; SD; 1 8 grams; average 64.2 mgm. % free, 16.9 mgm. % conjugated SD.

This monkey failed to recover from the effects of temporary dehydration during which the blood level rose to 55.9 mgm. % free and 11.8 mgm. % conjugated SD on the 11th day; gradual deterioration occurred and the animal was sacrificed on the 30th day of dosage in a moribund state, with a body temperature of 27°C. and a blood level of 74.1 mgm. % free and 36.9 mgm. % conjugated SD; weight—4.7 to 3.3 kgm.; Hb, 13.8 to 10.0 grams %; r.b.c., 5.5 to 3.4 millions. Kidneys—pale, capsule adherent, external surface sandy with deposits of drug; left kidney, small masses of crystals in calyces, crystalline deposit over entire cut surface of both kidneys; bladder and ureters—small amount of drug, no blockage. Microscopic findings: kidneys—granular, fibrinous and cellular casts in all collecting tubules, fibrous reaction surrounding many tubules, outlines of crystals in the tubules, glomeruli—fluid and debris in subcapsular spaces, thrombi in small renal veins; liver—fatty degeneration; adrenal cortex—pigmentation and hemorrhage in zona fascicularis; bone marrow—extreme fatty replacement, functional marrow very limited in amount.

Monkey #34; SD; 2.1 grams; average 32.8 mgm. % free, 4.4 mgm. % conjugated SD. This monkey recovered following a period of dehydration during which the blood level reached 59.9 mgm. % free and 9.9 mgm. % conjugated SD on the 11th day; this fell to 11.2 mgm. % free and 0.8 mgm. % conjugated SD on the 25th day; weight—1.9 to 1.7 kgm. in 10 days, 1.8 kgm. when sacrificed at end of 30 day period; Hb, 14.1 to 10.8 grams % on 11th day, 11.4 grams % on 31st day; r.b.c., 5.9 to 4.5 millions on 11th day, 4.7 millions on 31st day. No significant gross lesions were found. Microscopic findings: liver—edema and fat droplets in cells.

Monkey #37; SD; 2.4 grams; average, 49.0 mgm. % free, 7.2 mgm. % conjugated SD. This monkey recovered following a period of dehydration during which the blood level reached 64.7 mgm. % free and 8.2 mgm. % conjugated SD on the 11th day; this fell to 34.5 mgm. % free SD on the 25th day, and 34.8 mgm. % free and 0.2 mgm. % conjugated SD on the 29th day; weight—no change; Hb, 13.2 to 12.4 grams % on 11th day, 9.8 grams % on 31st day; r.b.c., 5.2 to 4.8 millions on 11th day, 4.0 millions on 31st day. No gross pathological changes were found. Microscopic findings: bone marrow—moderate fatty replacement; kidneys—slight interstitial edema, occasional leucocytes and epithelial casts in the renal tubules; liver—edema.

Monkey #31; SM; 0.5 gram; average, 46.4 mgm. % free, 42.4 mgm. % conjugated SM. This monkey died on the 15th day having failed to recover from dehydration; earlier on that day an 8 hour blood level was 43.3 mgm. % free and 62.7 mgm. % of conjugated SM; weight—3.5 to 3.1 kgm.; Hb, 11.4 to 10.1 grams %; r.b.c., 4.4 to 3.8 millions. The monkey was lethargic from the 8th day on, becoming progressively weaker until death; on the 11th day, and subsequently, intention tremors were noted. Kidneys—swollen and edematous, cortico-medullary relationship lost, crystal deposition throughout; bladder—numerous submucosal hemorrhages; no concretions in ureters or bladder. Microscopic findings: kidneys—granular and cellular casts in collecting tubules, plugs of fibrin and cells in convoluted tubules, outlines of crystals in the tubules, some hyperemia and occasionally fluid beneath Bowman's capsule, glomeruli—essentially normal, thrombi in small renal veins; liver—passive congestion, fatty infiltration and some edema, otherwise normal; bone marrow—considerable hyperemia and fatty replacement; ureters—a few areas of desquamation.

Monkey #33; SM; 0.6 gram; average, 51.4 mgm. % free, 39.6 mgm. % conjugated SM. This monkey died on the 16th day having failed to recover from dehydration; on the 15th day an 8 hour blood level was 78.6 mgm. % free and 57.4 mgm. % conjugated SM. Regurgitated twice following intubation during the 15 day period (reinjected); weight—1.9 to 1.7 kgm.; Hb, 13.5 to 10.8 grams %; r.b.c., 5.6 to 5.0 millions. Kidneys—soft, the surface gritty, pale and mottled, cortico-medullary relationship partially effaced and cut surfaces showed many crystals; left ureter—completely blocked with crystals for a distance of 5 cm. from the vesicoureteral junction; bladder—a few punctate sub-mucosal hemorrhages. Microscopic findings: identical with those of monkey #31; in addition, foci of polymorphonuclear leucocytes were found in the kidneys; adrenal cortex—hemorrhages in the zona fascicularis; ureters—no pathological change.

Monkey #35; SM; 0.7 gram; average, 26.9 mgm. % free, 2.5 mgm. % conjugated SM. This monkey survived the period of dehydration, and attained a peak blood level of only 35.6 mgm. % free and 1.1 mgm. % conjugated SM on the 11th day; this fell steadily to a level of 22.8 mgm. % free and 0.6 mgm. % conjugated SM on the 29th day. Regurgitation following intubation occurred 6 times during the 30 day period (reinjected); weight—no change; Hb, 14.5 to 10.9 grams %; r b.c., 4.3 to 3.8 millions. The plasma protein fell from 8.95 to 5.4 grams %. No gross evidence of pathological change was found. Microscopic findings; lungs—cellular infiltration from parasites; colon—cysts of parasitic infestation. Kidney and liver were normal in appearance.

Series 4.

Monkey #43; SD; 2.4 grams for 10 days, then, after 48 hours without drug, SD administration was resumed with 1 8 grams per kgm. per day; average blood concentration, 30 6 mgm. % free, 3.1 mgm. % conjugated SD; on the 10th day, 51.5 mgm. % free and 8.5 mgm. % conjugated SD; on the 28th day 13.8 mgm. % free, 0.5 mgm. % conjugated SD; weight —4.3 to 5 0 kgm.; Hb, 12.5 to 13.8 grams %; r.b.c., 5.1 to 5.2 millions. No gross pathological changes were found. Microscopic findings: kidney—slight intercapillary hyalinization in glomeruli; liver—foci of round cell infiltration, congestion and edema surrounding blood vessels with occasional areas of necrosis and parenchymatous degeneration; heart—small foci of round cell infiltration in epicardium and myocardium, also foci of round cells, macrophages and giant cells, resembling foreign body reaction; bone marrow—extreme fatty replacement in femurs and humeri; spleen—edema with reticular and lymphoid hyperplasia; thyroid—normal

Monkey #45; SD; 3.0 grams; average 46.8 mgm. % free, 11.7 mgm. % conjugated SD; survived. This animal developed edema and was seriously toxic within 10 days, when the blood level had risen to 69.3 mgm. % free and 31.7 mgm. % conjugated SD; spontaneous improvement occurred during the following week and by the end of the experimental period the monkey appeared to be in fairly good condition; weight—5.6 to 5.0 kgm.; Hb, 14.1 to 12.2 grams %; r.b.c., 5.5 to 4.1 millions. The animal appeared somewhat emaciated; lungs—numerous grayish-white nodules throughout, no caseation; kidneys—enlarged, pale, mottled, capsule adherent, rough and gritty external surfaces; the cut surfaces showed loss of cortico-medullary relationship and deposits of crystals throughout; there were extensive adhesions between the right kidney, colon, liver, peritoneum, omentum and body wall; liver—cyst on the anterior surface Microscopic findings: kidneys—severe tubular damage, epithelium hyperplastic or atrophic and degenerative, many casts; complete loss of parenchymatous structure in some areas, outlines of crystals in the tubules, glomeruli—some showed increased cellularity and numerous polymorphonuclear leucocytes, others normal, thrombi in small renal veins; liver—parasitic cyst, widespread necroses and tuberculoid lesions; lungs—round cell infiltration and tuberculosis, with little epithelioid response; heart—occasional foci of round cell infiltration; spleen—extreme hyperplasia and edema; thyroid—normal.

Monkey #[illegible]: SM 0 5 gram: average, 21.9 mgm. % free, 0.7 mgm. % conjugated SM; the hi[illegible] conjug[illegible] day. [illegible] —3.6 to 3.8 kgm ; Hb, 11.8 to 10.7 grams %; r b.c , 4.3 to 3.8 millions. No gross pathological changes were fou[illegible] active bone marrow and [illegible]

Monkey #44; SM; 0.6 [illegible] age, 20.8 mgm. % free, 1 [illegible] was 27.7 mgm % free, with no conjugated SM found; on the 28th day the blood level was 20.2 mgm. % free, with no detectable conjugated SM. This animal remained in excellent health throughout the entire 30 day period; weight—4.8 to 5.4 kgm.; Hb, 12.6 to 14.8 grams %; r.b.c., 5 8 to 5.7 millions No gross pathological changes were found. Microscopic findings; kidneys—possibly a trace of edema and slight intercapillary hyalinization in

glomeruli, otherwise entirely normal; lungs—early lesions of tuberculosis; heart—small epicardial tubercle; spleen—lymphoid hyperplasia; bone marrow—some fatty replacement; thyroid—normal.

Monkey #36; Control; survived and well; weight—2.0 to 2.3 kgm. during a 55 day period; Hb, 12.7 to 14.9 grams %; r.b.c., 4.9 to 5.2 millions. No gross or microscopic pathological changes were found.

STUDIES ON ABSORPTION AND EXCRETION. Shannon (26) has found from studies of the distribution of sulfamerizine and sulfadiazine in the various tissues of the cat that the two drugs follow essentially the same distribution pattern. This indicates that the difference in concentration in the blood produced by equal doses of the two drugs is essentially a reflection of dissimilar rates of absorption and urinary excretion. Blood concentrations are not influenced appreciably by variations in the distribution of the two drugs in the tissues. Shannon has also studied the ratio of the clearance of various sulfonamides to that of creatinine in dogs. It was found that the rate of excretion of SM by the dog kidney is approximately one half that of SD. Using a different method Marshall (27) has obtained results with dogs which led to a similar conclusion. The data herein presented, although obtained in different species, indicate that the higher concentrations in the blood produced by SM, in comparison with SD, are influenced by the slower rate of renal excretion of the methyl derivative. It would appear that the more rapid rise in sulfonamide concentration in the blood, following dosage with SM, may be attributed primarily to more rapid absorption, while the maintenance of a higher blood level is a result of not only a more complete absorption from the gastrointestinal tract but also a slower excretion of the drug by the kidneys. Since smaller doses of SM than of SD are required to produce a given concentration in the blood, and since the former drug is somewhat more soluble in urine below pH 7, it appears that the possibility of precipitation of sulfonamides in the urinary tract should be less with sulfamerizine.

Proof that the absorption of SM is more complete than that of SD requires the measurement of the total absorption of the drugs over a period of several days. This might be accomplished by the determination of the total urinary sulfonamide output or by the measurement of the total fecal excretion of unabsorbed sulfonamide. Both methods assume that insignificant amounts of absorbed drug are re-excreted into the lumen of the intestine, or are lost by other means from the body. Urinary excretion studies in man are described in a later portion of this paper. There it will be shown that even a small single dose of SM is absorbed more rapidly and more completely and is excreted more slowly than is SD. In monkeys the problem is more difficult.

The continued collection of urine from the monkey, without either loss or fecal contamination, is impractical when it is necessary to catch the animals every 8 hours for intubation. Accordingly, during a seven day period toward the end of the thirty day experiment, the well-formed feces of monkeys #43 (SD) and #44 (SM) were collected in entirety for analysis. It seems reasonable to assume that the collection of all feces for a period of this length is adequate

to avoid error due to daily variations in absorption and elimination. Reference to the sulfonamide concentration in the blood of monkeys #43 and #44 on the 21st, 24th and 28th days of the experiment (table 3) indicates that for the most part the blood levels were quite similar during this period.

Method of determination of sulfonamides in feces. The fecal material was collected essentially quantitatively, dried in a vacuum desiccator over phosphoric anhydride, and weighed. After reduction to a fine powder in a mortar and very thorough mixing of all the daily

TABLE 6

Sulfonamide content of samples of desiccated feces (7-day collection) of monkeys in which the sulfonamide concentrations in the blood were similar (see table 3) and which received sulfamerizine (SM), 0.9 gram per kgm. daily, and sulfadiazine (SD), 1.8 grams per kgm. daily, in 3 divided doses at 8 hour intervals

MONKEY NUMBER	DRUG	WEIGHT OF SAMPLE	ADDED DRUG	SULFONAMIDE FOUND	RECOVERY OF ADDED SULFONAMIDE	PERCENTAGE RECOVERY
		mgm	*mgm.*	*mgm.*	*mgm.*	
43	SD	100.0		18.56		
		100.0	2.0	20.42	1.86	93
44	SM	100.0		9.44		
		100.0	1.0	10.38	0.94	94

TABLE 7

Sulfonamide content of desiccated feces (7-day collection) of monkeys described in table 6, with data on the comparative absorption of sulfamerizine (SM), 0.9 gram per kgm. daily, and sulfadiazine (SD), 1.8 grams per kgm. daily, in 3 divided doses at 8 hour intervals

MONKEY NUMBER	DRUG	WEIGHT OF MONKEY		WEIGHT OF DRIED FECES	DRUG CONTENT OF 100 MGM. FECES	DRUG EXCRETED IN FECES	7-DAY DRUG INTAKE	DRUG ABSORBED FROM GASTRO-INTESTINAL TRACT
		Initial	Final					
		kgm.	*kgm.*	*grams*	*mgm.*	*grams*	*grams*	*grams*
43	SD	4.3	5.0	160.9	18.56	29.9	54.2	24.3
44	SM	4.8	5.4	93.5	9.44	8.83		
				10.1	8.26	0.83		
				7.7	6.52	0.50		
				111.3		10.2	30.2	20.0

specimens, aliquots of 100.0 mgm each were taken for analysis. The samples were placed in an Erlenmeyer flask and heated with 100 cc. of *N* NaOH at 100°C. for 2 hours. After cooling, the undissolved material was removed by filtration, the filtrate and washings neutralized with 4 *N* HCl and the volume adjusted to 200 cc Depending on the approximate sulfonamide concentration, estimated from a preliminary analysis, a suitable dilution of the filtrate was prepared with water and sufficient p-toluenesulfonic acid was added to give a concentration of 4 per cent. If not completely water-clear the diluted solution was filtered. Diazotization and coupling with *N*-(*1*-naphthyl)-ethylenediamine was carried out in the usual manner (21, 22) and the color produced was compared with those of standard solutions and of blanks simultaneously prepared

The results of the fecal analyses, shown in tables 6 and 7, indicate clearly that sulfamerizine is more completely absorbed than is sulfadiazine under the conditions of the experiment. The *absolute* amount of SM absorbed by monkey #44 (weight, 4.8 kgm.) was 20 grams, while 24.3 grams of SD were absorbed by monkey #43 (weight, 4.3 kgm.); the unabsorbed SM was only 10.2 grams, while 29.9 grams of SD passed through the gastrointestinal tract without absorption.

Unfortunately recovery was not made of the feces of monkey #42, which received only 0.5 gram of SM per kgm. daily, since the concentration of sulfonamides in the blood of this animal was also closely similar to that of monkeys #43 and #44. This monkey (#42) received only 12.6 grams of SM during the seven day period; absorption, therefore, must have been much more complete, even considering the weight difference (3.6 kgm.) and possible differences in the rate of renal excretion.

Data are not available to show what percentage of ingested SD would be excreted in the feces had the two drugs been administered in identical dosage. Under such conditions the concentration of SD in the blood would have been considerably lower than that produced by SM and the proportion of drug absorbed from the alimentary tract might have been somewhat different. However, data obtained in human beings, following the ingestion of equal doses (0.05 gram per kgm.) of the two drugs, show that a somewhat larger amount of SM is absorbed and excreted in the urine (table 9). The fecal excretion studies demonstrate that the very much larger dose of sulfadiazine necessary to produce a blood concentration equal to that produced by a given dose of sulfamerizine results in the absorption of somewhat similar amounts of the two drugs and, accordingly, a very much greater loss of sulfadiazine in the feces.

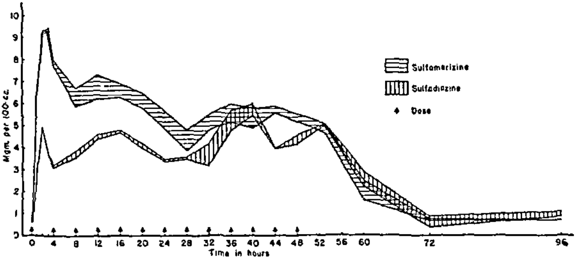
With increasing dosages of the two drugs the sulfonamide concentration in the blood rises much more strikingly with sulfamerizine than is the case with sulfadiazine. However, even within the range of dosage which might be employed in the clinical administration of the two drugs higher blood levels are produced with sulfamerizine. Figures 2 and 3 show the sulfonamide concentrations in the blood resulting from the initial administration to monkeys of 0.05 gram per kgm. of SM and of SD, followed by 0.018 gram per kgm. every 4 hours (0.1 gram per kgm. per day) for 48 hours. A two week period intervened between the two series of dosages presented in the figures.

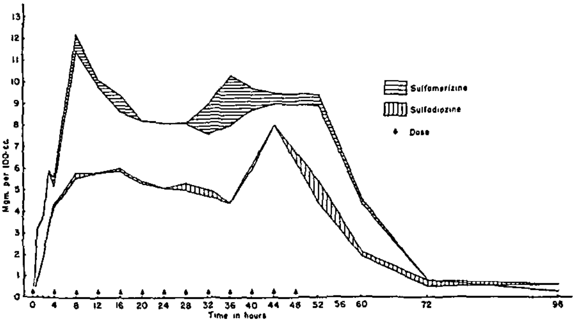
In man even smaller doses gave a similar result. In table 8 is shown the effect of the administration to a group of normal men of each of the two drugs in doses of 0.0125 gram per kgm., followed by 0.004 gram per kgm. every 4 hours for 48 hours.

Evidence that the difference in the rate of absorption of the two drugs is an important factor in the production of the higher and the more rapidly attained concentration of sulfamerizine in the blood is offered by the following experiment.

Absorption of SM and SD in bilaterally nephrectomized mice. The operated animals may be considered as relatively normal during the four hour period which is particularly important to the data presented; the average survival time of nephrectomized mice is greater than 24 hours.

About one hour following nephrectomy under light ether anesthesia, each of 5 mice was given sulfamerizine (10 mgm.) by mouth, in 0.5% tragacanth sus-





in the blood of a monkey (# 41) by o weeks, by sulfadiazine (SD). In each · free sulfonamide and the upper line the Initial dose: 0.05 gram per kgm. Subsequent doses: 0.018 gram per kgm. every 4 hours, last dose at 48 hours.

pension (0.5 cc.); a similar group received sulfadiazine in the same dose and by the same technic. The data are presented graphically in figure 4. It will be seen that within one hour the concentration of SM in the blood was approximately twice that of SD, while at the 4 hour and 20 hour intervals following

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Evidence that the difference in the rate of absorption of the two drugs is an important factor in the production of the higher and the more rapidly attained concentration of sulfamerizine in the blood is offered by the following experiment.

Absorption of SM and SD in bilaterally nephrectomized mice. The operated animals may be considered as relatively normal during the four hour period which is particularly important to the data presented; the average survival time of nephrectomized mice is greater than 24 hours.

centrations, the total absorption and the elimination of sulfamerizine and of sulfadiazine, a group of 14 normal human males was given the drugs at weekly intervals. Because of recent clinical interest in sulfamethazine (*2*-sulfanilamido-*4*,*6*-dimethylpyrimidine) (28, 29) this drug was also included in the study.

The drugs were given orally in powdered form, followed by a glass of water; the subjects were not informed as to the identity of the drug, each of which was administered in a dose of 0.05 gram per kgm. Blood samples were taken at the following intervals after dosage: 1, 2, 4, 8, 12, 24, 48 and 72 hours. In addition, each subject collected his total urinary output for 96 hours following dosage; the output was divided into the following periods: 0–8, 8–24, 24–48, 48–72, and 72–96 hours. Following dosage the only subjective symptoms of any

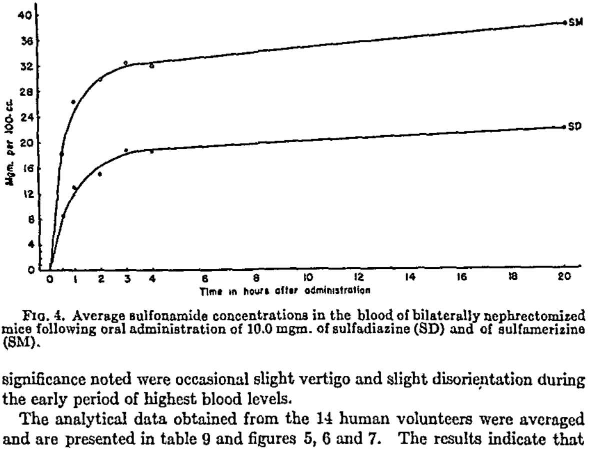


FIG. 4. Average sulfonamide concentrations in the blood of bilaterally nephrectomized mice following oral administration of 10.0 mgm. of sulfadiazine (SD) and of sulfamerizine (SM).

significance noted were occasional slight vertigo and slight disorientation during the early period of highest blood levels.

The analytical data obtained from the 14 human volunteers were averaged and are presented in table 9 and figures 5, 6 and 7. The results indicate that the value of the rapid absorption of sulfamethazine is to a certain extent nullified by the rapid urinary excretion of the drug. The evidence would suggest that rather frequent dosage would be necessary to maintain high blood concentrations. Macartney, *et al.* (28) found that a concentration of approximately 8 mgm. % was usually attained in from one to three hours after the oral administration of 4 grams in man. On dosage with 4 grams, followed by 2 grams every six hours, the blood level was extremely variable, as between individual cases, ranging from 2 to 13.5 mgm. %; the average blood level found by these workers was only 6 mgm. %.

Reference to figure 5 will show that following dosage with sulfamerizine a

concentration in the blood equal to that produced by sulfamethazine was almost as quickly attained, while the blood level produced by the same dosage of sulfadiazine was only about one half as great and was reached more slowly.

TABLE 9

Average urinary excretion of sulfamerizine (SM), of sulfadiazine (SD) and of sulfamethazine (SMZ) following the administration of 0.05 gram per kgm. of drug, as a single dose, to each of 14 normal men

DRUG	AVERAGE DOSE	TOTAL EXCRETION IN 96 HOURS	DRUG EXCRETED DURING 24 HOURS	DRUG IN TISSUES†		
				After 24 hours	After 48 hours	After 72 hours
	grams	*grams*	*grams*	*grams*	*grams*	*grams*
SM	3.58	2.78 (78%)*	1.33	1.45	0.54	0.15
SD	3.58	2.36 (66%)*	1.40	0.96	0.26	0.11
SMZ	3.58	3.04 (85%)*	2.30	0.74	0.14	0.0

* Percentage of total ingested dose which appeared in urine during 96 hours.

† Based on the assumption that essentially all absorbed drug appeared in the urine within 96 hours.

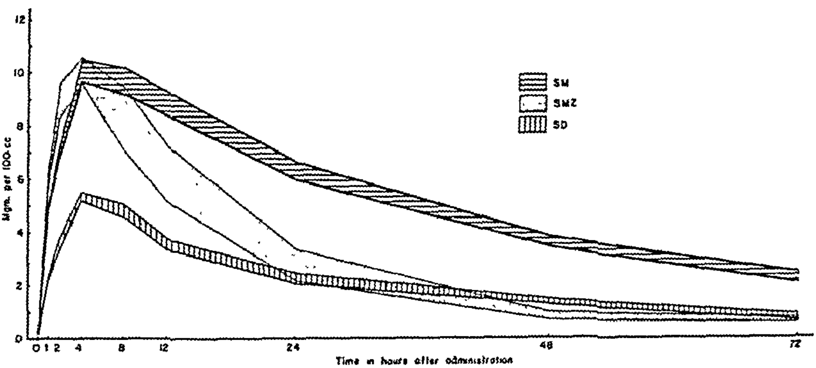
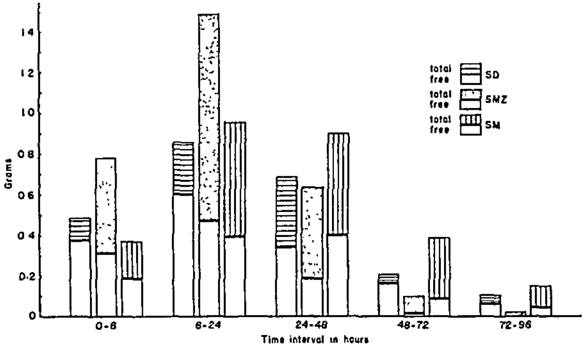


FIG. 5. Average concentration of sulfonamides in the blood of fourteen normal men following single oral doses of 0.05 gram per kgm. of sulfadiazine (SD), of sulfamerizine (SM) and of sulfamethazine (SMZ), alternately at intervals of one week. In each curve the lower line indicates the concentration of free sulfonamide and the upper line the concentration of total sulfonamide in the blood.

The data in table 9 and figures 6 and 7 suggest that the rate of renal excretion of SM in man is significantly less than is that of the other two pyrimidine derivatives, since the SM concentration in the blood fell so gradually from the peak attained 4 hours following dosage. Also, it will be noted (figure 7) that the absolute amount of SD excreted in the urine during the first 24 hours actually

exceeded the amount of the monomethyl derivative, despite the fact that the concentration of SD in the blood reaching the kidney was very much lower



in fourteen normal men at suc-
m per kgm. of sulfadiazine (SD),

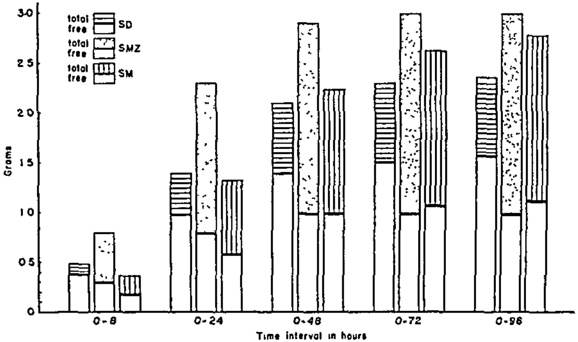


FIG. 7. Average cumulative urinary excretion of sulfonamides in fourteen normal men following single oral doses of 0.05 gram per kgm. of sulfadiazine (SD), of sulfamethazine (SMZ), and of sulfamerizine (SM).

than was the case with SM. It would be interesting to study the urinary excretion under conditions where equal blood levels were present; the data suggest that

to produce such a concentration in the blood it would be necessary to administer approximately twice as large a dose of sulfadiazine as is necessary with sulfamerizine. Under such circumstances the amount of sulfonamide which would have to pass through the kidneys would obviously be greater in the case of sulfadiazine.

Attention may be called to the urinary excretion of greater amounts of acetylated sulfonamide with the two methylated compounds, than is the case with sulfadiazine. The importance of the excretion of a smaller amount of acetylated sulfadiazine in the urine should not be emphasized, however, since the concentration of sulfonamide in the blood following the administration of sulfadiazine was so much lower than that produced by the other drugs. Furthermore, it should be stressed that so long as the degree of acetylation is not extensive enough to affect significantly the concentration of the free, active drug in the blood, *acetylation actually favors safe urinary excretion*. In the case of sulfamerizine it is distinctly beneficial to have a large proportion of the drug delivered to the kidney tubule in the more soluble, acetylated form. Reference to figure 1 will emphasize the significantly greater solubility of acetyl-SM in comparison with SM.

These observations, made on human beings, confirm and extend those made on other animals. It is shown clearly that in man sulfamerizine is rapidly absorbed and slowly excreted. The data show that following a dose of 0.05 gram per kgm. a sulfonamide concentration of 6 mgm. or more per 100 cc. of blood is maintained for 24 hours. These properties suggest that it may be possible to use sulfamerizine successfully in clinical practice on the basis of one or two doses daily. The rapidity with which the drug enters the blood stream of man indicates that it will rarely be necessary to resort to the intravenous injection of sodium sulfamerizine in order quickly to attain high blood levels.

STUDIES ON THE NERVE-DAMAGING POTENTIALITY OF SULFONAMIDES. It is well known that sulfonamides, like many other chemical substances, possess to a greater or lesser degree a potentiality for inducing nerve damage. Bieter, *et al.* (17) have reviewed the clinical literature and describe the situation obtaining with many of the more important sulfonamides introduced prior to sulfadiazine. It is interesting that sulfamethylthiazole (2-sulfanilamido-4-methylthiazole) is apparently much more frequently the cause of nerve lesions in human beings than is sulfathiazole (16). It is clear, however, that sulfathiazole (16, 30), sulfapyridine (31) and sulfadiazine (16) are capable of causing so-called peripheral neuritis in man, although the incidence has been extremely low. Consequently, inquiry as to whether or not sulfamerizine might possess the nerve damaging property to any degree directed our attention to the desirability of an experimental approach to the problem.

Bieter, *et al.* (17), following the lead of Rosenthal (18) and Nelson (19), reported that the chicken appears to be much more sensitive to the toxic effects of sulfonamides on the nervous system than is man, and further, that the severity of the nervous lesions observed in the chicken presents a rough parallel with the trend of clinical experience. The technic described by Bieter and his co-workers (17) has been employed in the following experiments with chickens.

A preliminary experiment with White Leghorn cockerels averaging 935 grams in weight yielded results of little value. In this test four sulfanilamide derivatives were administered to the birds: sulfamerizine, sulfadiazine, sulfamethylthiazole and sulfamethazine (*2*-sulfanilamido-*4*,*6*-dimethylpyrimidine). The last drug was given for two reasons, one, because of recent clinical interest in the compound, and two, because the presence of two methyl groups on the pyrimidine ring offered additional opportunity for study of the effect of methylation. Sulfamethylthiazole was used because this compound produced a relatively high incidence of neuropathy following its use both in human beings (16) and in chickens (17). The drugs were given by capsule once daily in dosages of 1.0 gram per bird, except in the case of sulfamethylthiaozle which was given in a dosage of 0.5 gram per bird. Within a week many of the experimental birds appeared acutely ill, while a control group remained healthy and gained weight. During the test all the birds on sulfamethazine died. Since microscopic study of sections of sciatic nerve and spinal cord disclosed no significant lesions in any of the birds, extensive data on the sulfonamide concentrations in the blood need not be presented here.

A second experiment was then designed in which the same sulfonamides were given for a period of 12 days to a group of cockerels (Barred Rock) averaging 770 grams in weight. The birds were kept two in a cage and fed Purina Growena *ad libitum*; the doses, which were administered by capsule *every 12 hours*, are shown in table 10; this table also presents other data significant to the experiment.

It should be noted that the birds on sulfamethylthiazole (SMT) all lost weight and 4 of 6 died. The two surviving cockerels presented some signs suggestive of neurological damage, although it is difficult to dissociate these from the general signs of depression associated with high tissue concentrations of sulfonamide. Among the signs were leg weakness, loss of balance, and occasional inability to walk. The animals on sulfadiazine (SD) either gained weight or remained constant in weight without manifesting signs of injury to nervous tissue, yet microscopic examination of the tissues from these birds revealed damage of the sciatic nerve in all cases and minor changes in the spinal cord of 2 birds. The finding of damage to nervous tissue, in the absence of observable signs, was also noted by Bieter and his group.

The birds on the lower dose of sulfamerizine (SM), 0.25 gram every 12 hours, all gained weight during the test and survived; the blood levels, although variable, were similar to those on the higher dose of SD and to those on the two dosages of SMT. On the higher dosage of SM, 0.5 gram every 12 hours, the drug concentration in the blood reached very much higher levels, but the neuropathological findings were neither more severe nor more frequent than those found in the birds which received the lower dosage of SM or the higher dosage of SD.

Bieter and his coworkers (personal communication) have also found sulfamerizine to be less damaging to the nervous tissue of the chick than either sulfadiazine or sulfathiazole.

No true correlation can be drawn between the high concentrations in the blood produced by sulfamethazine (SMZ) and the variable degree of nerve injury

TABLE 10

The effect of sulfamethylthiazole (SMT), of sulfadiazine (SD), of sulfamerizine (SM) and of sulfamethazine (SMZ) on the weight, on the concentration of sulfonamides in the blood, and on the development of neuropathologic changes in cockerels (Barred Rock) during a 12 day period

NUMBER OF BIRD	DRUG	INITIAL WEIGHT	CHANGE IN WEIGHT	DOSE PER 12 HOUR INTERVAL	DOSE PER KGM. PER DAY	CONCENTRATION OF SULFONAMIDE IN MGM. PER 100 CC. BLOOD: 7th day, 4 hours, Free	7th day, 4 hours, Conjugated	7th day, 12 hours, Free	7th day, 12 hours, Conjugated	12th day, 12 hours, Free	12th day, 12 hours, Conjugated	NEUROPATHOLOGICAL FINDINGS: Cord	Sciatic nerve	REMARKS
		grams	*grams*	*grams*	*grams*									
751	SMT	770	−320	0.25	0.65	16.8	5.5	20.7	4.6					Died on 11th day.
752	SMT	660	−270	0.25	0.76	11.8	2.8	15.0	4.4					Died on 7th day.
767	SMT	880	−370	0.25	0.57	17.1	0.6	18.7	2.3	17.6	6.6	No damage	Damage	Survived. Kidney—severe damage.
754	SMT	770		0.5	1.30	19.1	5.2	18.7	4.0			No damage	Damage	Killed on 9th day in extremis. Kidney—severe damage.
762	SMT	850	−350	0.5	1.18	22.8	9.1	22.6	3.3	19.0	1.5	No damage	Damage	Survived.
763	SMT	710		0.5	1.41	23.5	5.8	26.1	4.6			Damage	Damage	Killed on 9th day in extremis. Kidney—severe damage.
775	SD	820	+390	0.25	0.61	5.4	2.7	1.6	0	2.1	0	No damage	Damage	Survived. Kidney—norma l.
770	SD	720		0.25	0.69	11.7	8.7	11.4	0.6				Damage	Died during night—9th day. Kidney—damage.
778	SD	840	+360	0.25	0.60	13.6	1.7	3.2	0.1	4.7	0.2	No damage	Damage	Survived. Kidney—damage.
757	SD	700	+390	0.25	0.71	7.5	0.0	2.4	0.1	2.2	0.3	No damage	Damage	Survived.
768	SD	840	0	0.5	1.19	33.3	5.0	19.0	0	39.0	0.1	Damage	Damage	Survived. Kidney—severe damage.
779	SD	700	+120	0.5	1.43	36.8	3.8	28.9	0.1	18.0	0.8	No damage	Damage	Survived. Kidney—damage.
769	SD	760	+240	0.5	1.32	19.7	0	8.5	0	19.5	1.6	Damage	Damage	Survived. Kidney—damage.
780	SD	620	+190	0.5	1.22	19.3	0	9.1	0.1	5.9	0	No damage	Damage	Survived.
760	SM	820	+90	0.25	0.61	65.1	0	34.6	1.2	14.3	0	No damage	No damage	Survived.
755	SM	650	+150	0.25	0.77	32.2	0	10.0	0.7	13.1	0.3	Damage	Damage	Survived. Kidney—damage.
761	SM	810	+210	0.25	0.62	27.4	2.6	9.8	0.6	6.6	0.1	No damage	No damage	Survived.
765	SM	860	+90	0.25	0.58	36.7	8.6	14.4	0.3	14.6	0	No damage	Damage	Survived.
756	SM	750	+200	0.25	0.67	39.0	9.8	8.5	0.2	8.2	0.2	No damage	Damage	Survived.
777	SM	780	0	0.25	0.64	50.0	5.5	26.2	0.2	19.8	0.3	No damage	Damage	Survived. Kidney—severe damage.
761	SM	620		0.5	1.61	173.0	35.0					No damage	Damage	Killed on 7th day in extremis.
773	SM	850		0.5	1.18	82.5	5.6	70.7	4.0			No damage	Damage	Killed on 7th day in extremis. Kidney—normal.
774	SM	830	−90	0.5	1.20	85.4	2.9	80.5	3.9	16.3	0	No damage	Damage	Survived.
784	SM	780		0.5	1.28									Died during night 6th day.
770	SM	820	−100	0.5	1.22	89.0	0	78.2	1.3	12.6	0.4	No damage	No damage	Survived. Kidney—severe damage.
772	SM	760		0.5	1.32	93.6	5.6							Died during night—7th day.
753	SMZ	780	+90	0.25	0.64	62.5	1.6	41.9	2.8	19.5	0	No damage	No damage	Survived.
782	SMZ	600		0.25	0.63	102.0	4.0	95.3	13.6			No damage	No damage	Killed on 9th day in extremis. Kidney—normal.
759	SMZ	680	+50	0.25	0.74	62.8	0.4	32.5	1.7	46.2	0.6	No damage	Damage	Survived. Kidney—damage.
783	SMZ	870	−130	0.25	0.57	90.2	10.8	60.0	10.2	39.7	1.8	No damage	Damage	Survived.
786	Control	540	+270											All survived and well, no lesions in cord or sciatic nerve of #786 and #766, which were studied.
766	Control	960	+310											
764	Control	900	+130											
758	Control	610	+250											
771	Control	760	+160											
785	Control	790	+270											

found. Despite the high concentrations in the blood of chickens which received 0.25 gram of SMZ every 12 hours, and the presence of two methyl groups in the pyrimidine ring, sulfamethazine appears to possess no greater neurotoxic activity than sulfadiazine or sulfamerizine.

Our data, like those of Bieter, *et al.*, show that the chicken is inordinately sensitive to the nerve-damaging potentiality of sulfonamides. We have not felt justified, however, after discussion with consultant pathologists, in attempting to grade the severity of the neuropathologic lesions. It is possible that some attempt at gradation might be made on a basis of the comparative concentrations of sulfonamide produced in the blood of the birds receiving the various drugs. Clearly, the concentration of the methyl derivatives is consistently, and somtimes markedly, higher than that caused by sulfadiazine, yet nerve damage is, if anything, less frequent. The toxic effect of sulfamethylthiazole was great, despite blood levels which were not remarkably high, and only 2 of 6 birds survived the entire 12 day period. Although kidney specimens were not taken except from a few birds of each group, microscopic examination suggests that the renal tissue of chickens is readily damaged by sulfonamides.

The very low incidence of neuropathologic changes following the wide-spread use of sulfadiazine in human beings has been mentioned. The fact that sulfamerizine is at least as free of the neurotoxic property in the chick as is this well-known drug suggests that similar freedom from so-called peripheral neuritis may be anticipated with sulfamerizine.

DISCUSSION AND SUMMARY

A new sulfonamide, sulfamerizine (*2*-sulfanilamido-*4*-methylpyrimidine) has been studied extensively in various species (mice, rats, chickens, dogs, monkeys and men).

It produced no toxic manifestations when administered orally to white mice in doses as high as 32 grams per kgm. When given orally as the sodium salt the LD_{50} of the drug was approximately 2.5 grams per kgm. and all deaths occurred within 24 hours. With sodium sulfadiazine many deaths occurred after 24 hours; when *all* deaths are considered the LD_{50} was about 2.2 grams per kgm., but when only those deaths which occurred within 24 hours are used the LD_{50} of sulfadiazine was about 2.7 grams per kgm.

Paired-feeding experiments in rats indicated that the addition of sulfamerizine (0.5%) to the diet has no deleterious effect on the ability of the animals to utilize for weight gain the food which was ingested.

In dogs the continued administration of sulfamerizine in very large doses, 0.8 and 1.6 grams per kgm. daily, in three divided oral doses, resulted in the formation of drug concretions in the kidneys and varying depression of bone marrow activity in 5 of 6 animals (hyperplasia was noted in the sixth); focal necrosis and degenerative changes in the livers of some dogs were seen; these were marked in 2 of 3 animals on 1.6 grams per kgm. and slight in 1 of 3 animals on 0.8 gram per kgm. Since severe anorexia was noted in all these dogs, particularly those on the higher dose, and worm infestation was heavy, it is not considered fair to

state definitely that the drug was wholly responsible for the liver changes. Hepatic pathology was not seen in monkeys given comparable or even larger doses, or in dogs given 0.4 gram per kgm. *daily, in which anorexia was not present*. The last group of dogs received the drug during a period of 35 days without apparent toxic effects and neither gross nor microscopic evidence of tissue damage was seen, except for small concretions in the kidney pelves. Studies in monkeys, given doses similar to those received by the dogs, indicated that the sulfonamide concentration in the blood of the dogs on the higher doses probably ranged upwards from at least 30 mgm. % to zones exceeding 60 mgm. %. Since the dog does not acetylate sulfonamides it is probable that the material deposited in the kidneys consisted of free sulfamerizine.

Studies on the effect of prolonged administration to monkeys indicate that sulfamerizine and sulfadiazine are probably of similar toxicity when comparisons are made on the basis of the concentration in the blood. When sulfamerizine was given to healthy monkeys at 8 hour intervals in doses up to 0.6 gram per kgm. *daily*, the maximum average 8 hour concentration in the blood was 27 mgm. % (with a maximum of 2 to 3 mgm. % in the conjugated form). No evidence of tissue damage was seen in such animals. Sulfadiazine, given to monkeys in doses as high as 1.8 grams per kgm. daily (in three divided portions), usually produced lower average 8 hour concentrations in the blood than resulted from 0.6 gram per kgm. of sulfamerizine. The latter drug, in doses of 1.8 grams per kgm., caused sulfonamide concentrations in the blood of over 60 mgm. %. In monkeys we have not found evidence of toxicity, significant tissue damage of any kind, or kidney concretions, unless the average 8 hour concentration of sulfamerizine in the blood was over 30 mgm. %. The pathological findings in the 29 monkeys used in the chronic toxicity studies are described in detail in the text.

It is not possible to state precisely what blood concentration of either sulfamerizine or sulfadiazine is dangerously high. Toxic effects are probably dependent for the most part on precipitation of the drug in the kidney tubules or in more peripheral portions of the urinary tract. The occurrence of drug precipitation is dependent upon many factors other than drug concentration in the blood, particularly the extent to which the glomerular filtrate is concentrated during urine formation, the hydrogen ion concentration during the process, and the final volume and pH of the formed urine. Of great importance also is the solubility of the drug and its acetyl derivative in the urine. In this respect sulfamerizine is superior to sulfadiazine in neutral or acid urine (figure 1).

The fact that a much larger amount of sulfadiazine than of sulfamerizine must be administered in order to produce similar toxic concentrations in the blood and other tissues gives a false impression of the relative toxicity. For the maintenance of a stated high concentration in the blood much larger doses of sulfadiazine must be given because a larger amount of the drug escapes absorption and is excreted in the feces and because renal excretion of the absorbed sulfadiazine is more rapid.

Study of the effect of sulfamerizine and sulfamethazine in chickens has indicated that no greater potentiality for the production of neuropathologic changes

resides in these drugs than in the unmethylated pyrimidine derivative, sulfadiazine. Sulfamerizine, in much higher concentrations in the blood, was no more productive of lesions of the spinal cord and sciatic nerve in chickens than was sulfadiazine. Since the extensive clinical use of sulfadiazine has led to only one reported case of nerve damage it appears unlikely from our data that so-called peripheral neuritis will follow the clinical use of sulfamerizine.

Investigation of the mechanisms responsible for the higher blood concentrations produced by sulfamerizine, in comparison with sulfadiazine, disclosed that the drug is (*1*) more rapidly absorbed, (*2*) more completely absorbed, and, (*3*) more slowly eliminated by the kidneys. The fact that the distribution of sulfamerizine and sulfadiazine in the tissues is very similar (26) permits conclusions to be drawn concerning the roles of absorption and elimination. When renal elimination of the drugs was prevented by administration to bilaterally nephrectomized mice it was shown that the more rapid rise in the concentration of sulfamerizine in the blood, in comparison with sulfadiazine (figure 4), must result primarily from the more rapid absorption of the drug from the alimentary tract.

That the absorption of the methyl derivative is somewhat more complete than is that of sulfadiazine, was shown in two ways: (*1*) two monkeys were given the drugs in large doses every 8 hours, in amounts sufficient to produce comparable blood concentrations; all feces were collected in entirety and analyzed for total sulfonamide content. Of 54.2 grams of sulfadiazine administered during a period of one week (1.8 grams per kgm. daily), 29.9 grams appeared in the feces, indicating that 24.3 grams were absorbed. Of 30.2 grams of sulfamerizine given during the same period (0.9 gram per kgm. daily), 10.2 grams appeared in the feces, indicating the absorption of 20.0 grams (table 7); (*2*) administration of each drug to a group of 14 human subjects, in a single dose of 0.05 gram per kgm., and collection of all urine for 96 hours (figure 7) permitted the calculation of the average total amount of drug absorbed. Even with these small, equal, "clinical" doses the absorption of sulfamerizine was definitely superior to that of sulfadiazine: sulfamerizine, 78% and sulfadiazine, 66%.

The fact that sulfamerizine is excreted by the kidney more slowly than is sulfadiazine is indicated by the data presented herein. In addition, Shannon (26) found, from studies of the creatinine clearance ratios in dogs, that sulfamerizine is excreted at a rate approximately one-half that of sulfadiazine.

Administration of sulfamerizine to a group of 14 men showed (figure 5) that an average concentration of approximately 8 mgm. % can be produced in the blood within two hours, and of 10 mgm. % in 4 hours, by a dose of only 0.05 gram per kgm. (3.5 grams, or 53 grains, in a man of 150 pounds of body weight). A level greater than 6 mgm. % in the blood is maintained for 24 hours thereafter; this falls to about 3.5 mgm. % 48 hours after administration. When sulfadiazine was given to the same individuals in the same dosage an average concentration of approximately 3.0 mgm. % was produced in the blood within 2 hours, reaching a peak of 5 mgm. % in 4 hours. After 24 hours the blood level was reduced to about 2 mgm. %.

Although one of the factors responsible for the greater concentration in the blood following sulfamerizine administration is the more rapid absorption of the drug, much weight must also be placed on the relative rates of renal excretion. *Figure 7 shows that more sulfadiazine than sulfamerizine is excreted in the urine* during the first 24 hours, despite the fact that the blood reaching the kidney is very much lower in its sulfadiazine content. With equivalent clinical concentrations of the two drugs maintained in the blood it may be anticipated that, as a result of the slower renal excretion of the smaller amount of sulfamerizine, a definitely lower concentration of that drug should occur in the urine at any given time. This, together with the greater solubility of the free and acetylated forms of sulfamerizine in neutral or acid urine, in comparison with sulfadiazine, offers additional protection to the urinary tract from the possibility of formation of drug concretions.

The properties of sulfamerizine offer the definite possibility that an adequate concentration of sulfonamide in the blood and other tissues may be maintained by the administration of one or two doses daily.

The chemotherapeutic activity of sulfamerizine has been studied by our colleague, Dr. Willard F. Verwey. In a paper to be published experimental data will be presented which show that the absorption and excretion characteristics of sulfamerizine valuably influence the high chemotherapeutic activity of the compound.

The microscopic studies of tissues have been carried out with the aid and advice of Dr. Dale R. Coman, of the Department of Pathology, University of Pennsylvania School of Medicine. We are deeply indebted to Dr. Arnold R. Rich, of The Johns Hopkins University, who examined sections of the liver and kidney tissue of all monkeys, and who is in agreement with the statements relating to the findings in those tissues. We are grateful to Professor E. K. Marshall, Jr., of The Johns Hopkins University, and to Dr. James A. Shannon, of New York University, for opportunities to discuss these studies and for permission to quote from their unpublished work. We wish to thank Dr. Lawrence Peters and Dr. Earl L. Burbidge for their aid during a phase of the investigation.

CONCLUSIONS

The data presented indicate that in sulfamerizine a new chemotherapeutic agent is available which is more rapidly absorbed than sulfadiazine, which produces a high concentration in the blood with a smaller dose than with sulfadiazine, and which is more slowly excreted by the kidney in a form more soluble than sulfadiazine in neutral or acid urine. The acetyl derivative is more soluble than sulfamerizine and more soluble than acetylsulfadiazine in urine at a pH of 7 or less.

Prolonged administration to monkeys indicates that the two drugs are probably of similar toxicity when a comparison is made on the basis of the concentration in the blood. Studies in chickens indicate that the drug has no greater potentiality for the production of neuropathologic changes than has sulfadiazine.

The possibility is offered that one or two doses of sulfamerizine daily may be sufficient to maintain a safe and adequate concentration of sulfonamide in the blood and other tissues. The rapidity of absorption suggests that intravenous injection may not be necessary when it is desired to produce an adequate blood concentration quickly.

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THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND INHIBITORY ACTION OF BARBITURIC ACID DERIVATIVES ON RAT BRAIN RESPIRATION *IN VITRO*[1]

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The relationship between chemical structure and pharmacological action of a large number of barbiturates has been extensively investigated in the intact animal, where anesthesia and death may be used as criteria of intensity of action (1, 2, 3). Similar investigations of structural relationships have been made on the rate of hemolysis of red blood cells (4), and on the inhibition of the rate of cell division of *Arbacia* eggs (5). The work of Quastel and his associates (cf. 6) has shown that the barbituric acid derivatives inhibit the oxidation by brain tissue of glucose, lactate and pyruvate. The exact point of action of these inhibitors has not yet been established, but there is evidence indicating that the enzyme affected is either a flavoprotein functioning as a link between dehydrogenase and cytochrome, or an unknown component of the cytochrome system, a component which is not involved in succinate oxidation (7).

The effects of graded concentrations of three groups of substituted barbituric acid derivatives on the respiration of slices of rat cerebral cortex are described in this paper. These data supply a basis for quantitative comparison of the inhibitory action of the drugs, and for relating the inhibitory action to their physical properties and to their physiological actions on other systems. The data also supply some basis for comparing dosages which induce anesthesia *in vivo* with concentrations depressing brain respiration *in vitro*.

METHODS. Eighteen adult female albino rats were used. These were killed by decapitation and the brains were removed as quickly as possible. Slices of cerebral cortex, prepared as described previously (8), were placed in Warburg respirometer vessels containing Ringer's-phosphate-glucose solution at an initial pH of 7.3 (glass electrode). Because of the alkalinity of the sodium salts of the barbiturates there was an increase in pH when the drugs were added in the higher concentrations. Over most of the working range of concentrations the final pH did not exceed 8.0. Oxygen consumption was measured manometrically by the direct method of Warburg (9). Details of procedure peculiar to the problem in hand were these: Approximately 25 mgm. of fresh tissue were weighed on a microtorsion balance and placed in 0.9 cc. of buffered Ringer's solution in the respirometer vessel. This vessel contained in its sidearm the desired concentration of barbiturate dissolved in 0.1 cc. of buffered Ringer's. The vessels were well flushed with oxygen and equilibrated 15 minutes at 37.5 ± 0.01°C. before setting. The time between death of the animal and the beginning of the measurement of respiration was 30 minutes. Four readings were taken at 10 minute intervals before addition of the drug from the vessel sidearms. The control rate of oxygen consumption was determined from these measurements. It is expressed in terms of cubic millimeters, N.P.T., per milligram dry weight per hour ($Q_{O_2}C$).

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The barbiturate was then added from the sidearm and readings were taken at 15 minute intervals for 2 hours. Whenever inhibition of respiration occurred it was fully developed in 30 minutes. Therefore the rate of oxygen consumption in the presence of the inhibitor ($Q_{O_2}I$) was calculated from readings taken during the period 45 to 75 minutes after addition of the drug. The results are expressed as the ratio of the two rates, $\frac{Q_{O_2}I}{Q_{O_2}C}$, which is called the *inhibition ratio.*

The barbituric acid derivatives used were commercial preparations. The nomenclature used is that employed in current issues of *Chemical Abstracts.* The type structure of barbiturates according to this scheme is given in the paper of Clowes, *et al.* (5). All solutions were made up in buffered Ringer's and were used on the day of preparation.

RESULTS. *1. Concentration-action relationships.* The barbituric acid derivatives used are classified in three groups for convenience in discussion. Group I consisted of an homologous series of six *5*-alkyl-*5*-ethyl barbiturates, in which the alkyl substitution ranged from the ethyl to the hexyl group. Group II consisted of an homologous series of three *5*-allyl-*5*-alkyl derivatives in which the alkyl groups were isopropyl, isobutyl and *1*-methylbutyl respectively. Group III was rather miscellaneous, consisting of two cyclic derivatives and one thiobarbiturate. The compounds in these groups are listed in table 1.

The effect of graded concentrations of these barbiturates on the oxygen consumption of rat cerebral cortex *in vitro* is illustrated in figures 1, 2, 3 and 4. It is shown in figures 1 and 2, for the alkylethyl (Group I) and alkylallyl (Group II) series respectively, that the efficacy of the compound as an inhibitor of oxygen consumption increased with the length of the carbon chain in the alkyl groups. This generalisation does not apply to the compounds in Group III, the cyclic derivatives and the thiobarbiturate. In regard to the latter, introduction of a sulfur atom appeared to diminish the inhibitory potency of the compound, because the concentration of *5*-ethyl-*5*-(*1*-methylbutyl)-*2*-thiobarbiturate required to produce 50% inhibition was about four times that of the *5*-ethyl-*5*-(*1*-methylbutyl) barbiturate (cf. figure 3 and table 1).

Those derivatives which caused little inhibition in the concentration range covered by the data of figures 1 and 3 were studied at higher levels of concentration. The data so obtained are given in figure 4, in which the inhibition ratio is plotted as a function of the log of the molar concentration. It is shown in figure 4 that the highest concentration of *5,5*-diethyl barbituric acid used (corresponding to 1% of the sodium salt) produced only about 50% inhibition of brain respiration. Likewise with *5*-ethyl-*5*-isopropyl barbituric acid the maximum inhibition obtained was less than with the other drugs; a decrease in respiration of about 72% was found in the presence of an 0.8% solution of the sodium salt.

It is also shown in figure 4 that even in very high concentrations of the effective inhibitor, *5*-ethyl-*5*-(*1*-methylbutyl) barbiturate, there is a fraction of cerebral cortex respiration which is not inhibited. The tendency of the curves of figure 1 to become asymptotic to the X-axis also constitutes evidence of a fraction of brain respiration stable toward barbiturate inhibition. The data of figures 2, 3 and 4 are in harmony with this view. An inhibitor-stable fraction of the respiration of rat brain cortex has been observed in the presence of a

number of other agents depressing brain respiration such as propazone (10), diphenyloxazolidinedione (8), sodium azide and 2,4-dinitrophenol (11).

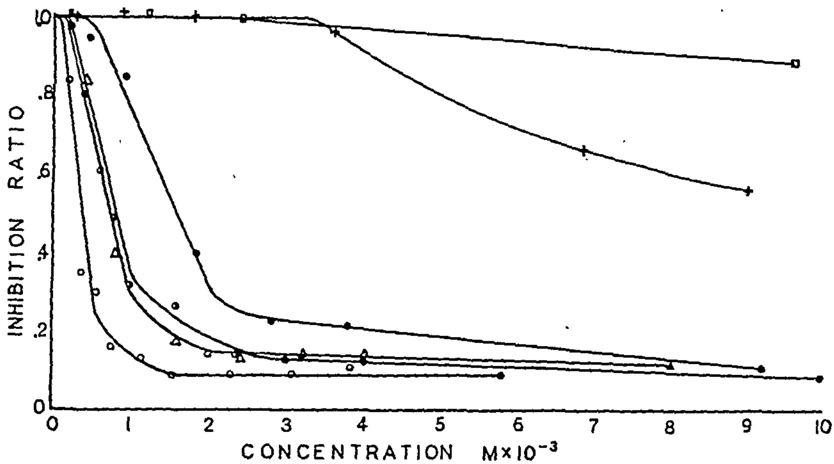

FIG. 1. EFFECTS OF GRADED CONCENTRATIONS OF 5,5-ALKYL-ETHYL BARBITURIC ACID DERIVATIVES ON THE OXYGEN CONSUMPTION OF RAT BRAIN CORTEX *in vitro*

□, 5,5-diethyl; +, 5-ethyl-5-isopropyl; ●, 5-butyl-5-ethyl; △, 5-ethyl-5-isoamyl; ◑, -ethyl-5-(1-methylbutyl); ○, 5-ethyl-5-hexyl.

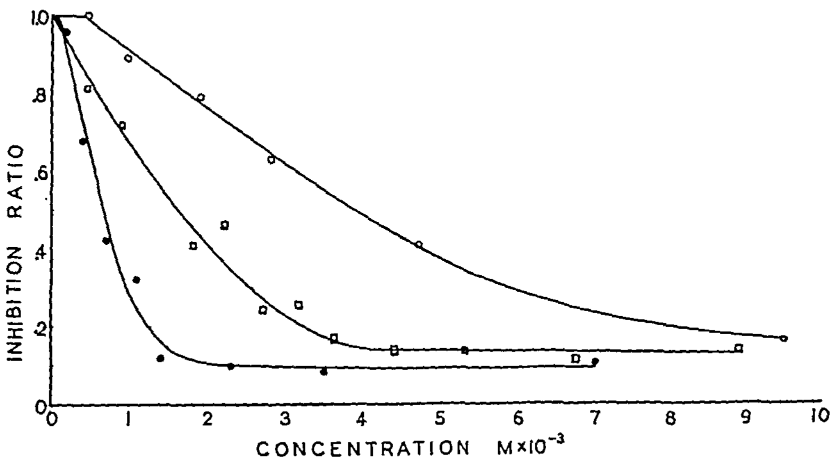

FIG. 2. EFFECTS OF GRADED CONCENTRATIONS OF 5,5-ALKYL-ALLYL BARBITURIC ACID DERIVATIVES ON THE OXYGEN CONSUMPTION OF RAT BRAIN CORTEX *in vitro*

○, 5-allyl-5-isopropyl; □, 5-allyl-5-isobutyl; ●, 5-allyl-5-(1-methylbutyl)

2. Parallelisms between physical properties and biological actions. It is shown in table 1 that there are some striking parallelisms between certain of the physical properties and the biological actions of the drugs in Groups I and II, the

alkylethyl and alkylallyl derivatives respectively. Thus with increasing length of the carbon chain in the alkyl substituent there are:

(*1*) Decrease in the molar concentration required to produce 50% inhibition of the oxygen consumption of rat cerebral cortex *in vitro*.

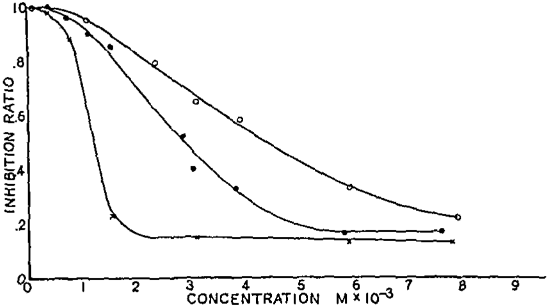

FIG. 3. EFFECTS OF GRADED CONCENTRATIONS OF TWO CYCLIC AND ONE THIO-BARBITURIC ACID DERIVATIVES ON THE OXYGEN CONSUMPTION OF RAT BRAIN CORTEX *in vitro*
○, *5*-ethyl-*5*-phenyl; ×, *5*-allyl-*5*-cyclopentenyl; ●, *5*-ethyl-*5*-(*1*-methylbutyl)-*2*-thio

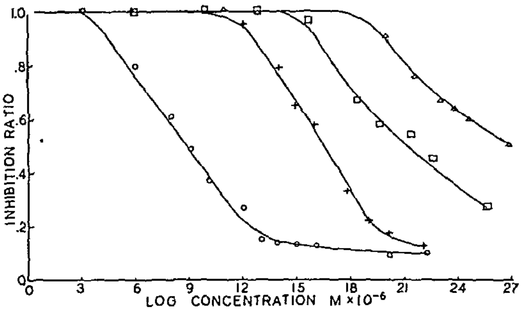

FIG. 4. EFFECTS OF HIGH CONCENTRATIONS OF SOME *5,5*-SUBSTITUTED BARBITURIC ACID DERIVATIVES ON THE OXYGEN CONSUMPTION OF RAT BRAIN CORTEX *in vitro*
△, *5,5*-diethyl; □, *5*-ethyl-*5*-isopropyl; +, *5*-ethyl-*5*-phenyl; ○, *5*-ethyl-*5*-(*1*-methylbutyl)

(*2*) Decrease in the molar concentration required to produce 50% inhibition of cell division of fertilized *Arbacia* eggs (5).

(*3*) Delay in the onset of anesthetic action on intravenous administration to mice (12).

(4) In general, decreasing solubility in water (13).
(5) In general, increase in the oil/water distribution coefficient (13).

3. Relationship between concentrations inducing anesthesia in vivo and inhibition of brain respiration in vitro. It is shown in table 2 for the compounds of Group I, the alkylethyl derivatives, that when the concentration of barbiturate in the suspension medium in the respirometer vessels was that which should be present in the intact animal on the assumption that the AD_{50} is uniformly distributed throughout the body, there was very little inhibition of brain respiration except in the case of the *5,5*-ethylhexyl derivative. However, when the concentration in the suspension medium was four times that predicted in the animal on the basis of uniform distribution, (cf. 14), all of the drugs but the first two caused notable diminution in respiration. The values of the inhibition ratio obtained under the latter conditions were also quite variable.

TABLE 1

Some relationships between physical properties and physiological action for three groups of substituted 5,5′-barbituric acid derivatives

Column I. Carbon atoms in alkyl side chain.
II. Concentration ($M \times 10^{-4}$) producing 50% inhibition of rat brain cortex oxygen consumption.
III. Concentration ($M \times 10^{-4}$) producing 50% inhibition of Arbacia egg cell division (5).
IV. Delay in onset of anesthetic action (in minutes) following intravenous administration to mice (12).
V. Solubility in water. Grams per liter (13).
VI. Oil/H_2O distribution coefficient (13).

	I	II	III	IV	V	VI
Group I. Alkyl-ethyl derivatives:						
5,5-diethyl	2	480.0	320.0	22.0	6.00	0.214
5-ethyl-*5*-isopropyl	3	130.0	162.0	9.2	1.36	0.73
5-butyl-*5*-ethyl	4	16.0	40.0	1.6	1.90	2.58
5-ethyl-*5*-(*1*-methylbutyl)	5	8.5	12.0	0.1	1.20	4.40
5-ethyl-*5*-isoamyl	5	7.5	15.0	0.2	0.53	2.89
5-ethyl-*5*-hexyl	6	4.0	7.5	0.0		
Group II. Alkyl-allyl derivatives:						
5-allyl-*5*-isopropyl	3	39.0	98.0	12.4	4.02	1.12
5-allyl-*5*-isobutyl	4	16.0	39.0	3.4		
5-allyl-*5*-(*1*-methylbutyl)	5	6.5	2.4	0.1		
Group III. Miscellaneous:						
5-ethyl-*5*-phenyl	(6)	43.5	95.0	12.3	0.97	1.34
5-allyl-*5*-cyclopentenyl	(5)	12.5		1.6		
5-ethyl-*5*-(*1*-methylbutyl)-*2*-thio	5	28.5				

Discussion. *1. Relationship between chemical structure and physiological action.* It is well established that, within limits, increase in length of one or both alkyl side chains in a barbiturate results in increased anesthetic potency

(2, 3, 15). It is shown in table 1 that for members of the two homologous series studied, the alkylethyl and alkylallyl derivatives, increase in the length of one side group resulted in a marked increase in the potency of the drug as an inhibitor of brain respiration. Thus the concentration of the *5,5*-diethyl compound required to cause 50% inhibition was 4.8×10^{-3}M., while only 4×10^{-4}M. of the *5*-ethyl-*5*-hexyl derivative had the same effect. There is a parallelism in the relationship between chemical structure and anesthetic action on the intact animal on the one hand, and the potency as an inhibitor of brain respiration on the other. Just how far the parallelism between Swanson's observations (2, 3) and ours obtains cannot be said. We found the *5*-ethyl-*5*-hexyl derivative more effective as an inhibitor of cerebral cortex respiration than either derivative with a five carbon atom side chain, whereas Swanson found that when

TABLE 2

Inhibition ratios (QO_2I/QO_2C) of rat brain respiration in vitro produced by concentrations of 5,5-alkyl-ethyl barbiturates corresponding to the AD_{50} and 4 times the AD_{50} for the intact rat

BARBITURATE	AD_{50}[1]	INHIBITION RATIO IN VITRO[2]	
		AD_{50}[3]	$4 \times AD_{50}$[4]
	mgm./kgm.		
5,5-diethyl	340	1.00	0.94
5-ethyl-*5*-isopropyl	161	1.00	0.98
5-butyl-*5*-ethyl	74	0.92	0.59
5-ethyl-*5*-(*1*-methylbutyl)	43	0.98	0.60
5-ethyl-*5*-isoamyl	80	0.95	0.23
5-ethyl-*5*-hexyl	89	0.59	0.10

[1] From Swanson (2) and Swanson and Fry (3) for intraperitoneal administration to albino rats.

[2] Calculated graphically from data given in figure 1.

[3] Assuming equal distribution of the AD_{50}.

[4] Assuming a tissue concentration four times that expected on the basis of equal distribution of the AD_{50}.

the length of the carbon chain in the alkyl group was increased above five, the dosage required to anesthetize or kill rose again.

The miscellaneous compounds in Group III, including the two cyclic derivatives and a thiobarbiturate, do not fit into the generalisation that potency as an inhibitor of brain respiration increases with length of a side chain. However they are not homologous and would not be expected to show graded properties (cf. 16). It is interesting that the introduction of a sulfur atom decreased inhibitory potency as shown by the comparison between *5*-ethyl-*5*-(*1*-methylbutyl)-*2*-thiobarbiturate and *5*-ethyl-*5*-(*1*-methylbutyl) barbiturate.

Two other comparisons between chemical structure and physiological action of substituted barbiturates are shown in table 1. Listed in column III are the concentrations required to produce 50% inhibition of the rate of cell division in fertilised *Arbacia* eggs. These data are taken from the work of Clowes, Keltch and Krahl (5). It is clear that not only is there a parallelism in the potency of

the compounds with respect to these two actions, but also that the "50% inhibition" concentrations are of the same order of magnitude, in spite of the difference in type of effect and in animal form. Given in column IV, table 1, are the latency periods found by Butler (12) for the induction of anesthesia in mice after intravenous administration of 1.25 times the AD_{50}. It is seen that in general these data also show systematic diminution in latency time with increase in length of the alkyl side chain.

2. Relationship between physical properties and physiological action. It has long been known that in various homologous series there is an increase in narcotic potency with decreasing solubility in water and increase in oil/water distribution coefficient (16, pp. 178, 189). Using data of Tabern and Shelberg (13) we have shown in table 1 that a similar parallelism obtains in the compounds constituting Groups I and II. The possible relationship of these physical properties to the mechanism of anesthetic action has been discussed in various reviews, including those of Winterstein (17), Henderson (16), Clark (18) and Meyer (19).

3. Relationship between concentrations inducing anesthesia in the intact animal and those depressing brain respiration in vitro. The observations reported here provide the most complete and precise data yet available concerning the action of barbiturates on brain respiration *in vitro.* Thus there is a favorable opportunity to compare the effects of these central nervous system depressants *in vitro* with their known actions *in vivo.* The relationship between anesthetic action, depression of brain respiration and the concentration of drug in the brain, cannot be formulated in quantitative terms until the independent variable, the concentration of drug in the brain, can be measured satisfactorily *in vivo* and *in vitro.* The present status of estimates of this factor may be summarized as follows: For the intact animal the concentration of drug in the brain has usually been estimated on the basis of uniform distribution of the dose administered, without correction for non-vascular tissue (20). The chief basis for this assumption in the case of the barbiturates is the work of Koppanyi and his associates (21, 22, 23), who reported that no organ or tissue in the dog or the rabbit has a special affinity for either *5,5*-diethyl- or *5*-ethyl-*5*-(*1*-methylbutyl)-barbiturate. According to their data however the concentration of the former drug was slightly higher, and of the latter slightly lower, in the brain than in other organs. There has been improvement in methods of assay since this work was done (24). Recently Tatum, Nelson and Kozelka (14), using improved methods, found that fifteen minutes after intravenous administration to rabbits of *5*-ethyl-*5*-isoamyl barbituric acid, the concentration in the brain was about four times that to be expected on the basis of uniform distribution. This rather high differential concentration decreased with time. Similar observations have been reported following administration of *5,5*-diethyl barbituric acid to an elephant (25). In a recent paper we presented evidence indicating that diphenyloxazolidinedione, which is structurally related to the barbiturates, shows differential concentration in the brain of the intact rat (8). Thus at present the weight of evidence favors the view that after administration of a barbiturate to the intact animal, the con-

centration of the drug in the brain rises for a time to a level greater than that predicted on the assumption of equal distribution.

Whether the distribution of the ureide hypnotics is uniform or differential in the system composed of suspension medium and cerebral cortex is not known. There is some evidence that the concentration gradient, if any, is small (8). Furthermore, the excised brain is deprived of certain channels of drug loss available in the intact animal, such as possible destruction in the liver (14, 26), excretion and other detoxication mechanisms. Since the oxygen consumption of excised brain soon becomes constant after addition of a barbiturate, and remains constant for over three hours, it appears that drug-cell equilibrium (cf. Clark-27) is attained and maintained, whether the concentration be uniform or differential.

It is shown in figures 1 to 4 that brain respiration is profoundly reduced by *various barbiturates in suitable concentrations. Profound depression of brain* respiration would surely result in anesthesia in the intact animal (6, 28). However it is not certain whether anesthesia is consequent upon moderate reduction of brain respiration, or whether these two effects are independent. Several possibilities may be stated. Anesthesia may be induced by:

(*1*) Concentrations of narcotic which do not affect brain respiration

(*2*) Concentrations of narcotic moderately reducing brain respiration to some common threshold level

(*3*) Concentrations of various narcotics depressing brain respiration to different levels.

The data of table 2 establish certain conditions which an acceptable hypothesis must take into account. Column 2 (headed "AD_{50}") shows the inhibition ratio of cerebral cortex respiration when the concentration of barbiturate in the suspension medium was the same as the AD_{50} for rats, calculated from the data of Swanson and Fry (3) on the assumption of uniform distribution. At this concentration appreciable inhibition of respiration occurred only with the ethylhexyl derivative. However, at four times this concentration level (a condition designed to simulate the differential concentration found by Tatum *et al.* (14)), all the barbiturates of Group I with more than 3 carbon atoms in the alkyl side chain caused appreciable inhibition, as shown in table 2, column 3. The significance of the diminishing value of the inhibition ratio with increase in length of alkyl side chain (table 2, column 3) is not clear. Possibly it implies that there is a progressive change in differential concentration of the drugs in the brain; possibly that the anesthetic action of these drugs develops at different levels of depression of brain respiration.

Further development of the relationships between anesthetic action and brain respiration will be feasible when more is known of the drug-cell equilibria involved.

SUMMARY

1. The effects of graded concentrations of three groups of substituted barbituric acid derivatives on the respiration of rat cerebral cortex slices were de-

scribed. Group I consisted on an homologous series of six 5-alkyl-5-ethyl barbiturates in which the alkyl substitution ranged from the ethyl to the hexyl group. Group II was composed of an homologous series of three 5-allyl-5-alkyl derivatives in which the alkyl groups were isopropyl, isobutyl and 1-methylbutyl respectively. Group III was miscellaneous, consisting of two cyclic derivatives and one thiobarbiturate. The data were presented in the form of concentration-action curves.

2. It was found that with increasing length of the alkyl sidechain in the compounds of groups I and II there was decrease in the molar concentration of the compound required in the suspension medium of the respirometer vessel to produce 50% inhibition of the oxygen consumption of rat cerebral cortex *in vitro*. Several other parallelisms between the physical properties and biological actions of these drugs were described from data in the literature.

3. The problem of the relationship between doses of these drugs inducing anesthesia *in vivo* and concentrations inhibiting brain respiration *in vitro* was discussed. It was pointed out that quantitative formulation of this relationship must await precise determination of the concentrations of the drugs in the brain, both *in vivo* and *in vitro*.

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SYNTHETIC GLYCOSIDES OF DIGITOXIGENIN, DIGOXIGENIN AND PERIPLOGENIN

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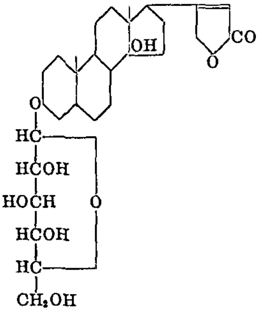
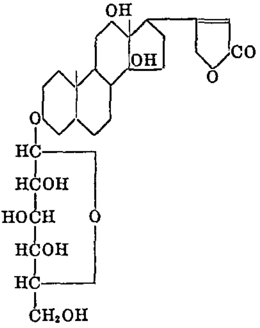
In previous reports (1, 2) the preparation of several new cardiac glycosides was described and their potency evaluated in cats and frogs. The outstanding feature of the results was that certain synthetic glycosides of strophanthidin were more active than the natural glycoside, cymarin. Chemical work has now been extended to digitoxigenin, digoxigenin, and periplogenin at the Department of Chemistry, Columbia University (3). By the reaction of one molecule of glucose with the secondary OH group on carbon atom *3* of each aglycone, glucosides of digitoxigenin, digoxigenin, and periplogenin were successfully synthesized. As before, the aglycone was first coupled with acetobromoglucose, and the resulting tetraacetyl-glucoside was then deacetylated. Thus,

Digitoxigenin + acetobromo-*d*-glucose $\longrightarrow$
Digitoxigenin-β-tetraacetyl-*d*-glucoside $\longrightarrow$
Digitoxigenin-β-*d*-glucoside + 4 CH_3COOH

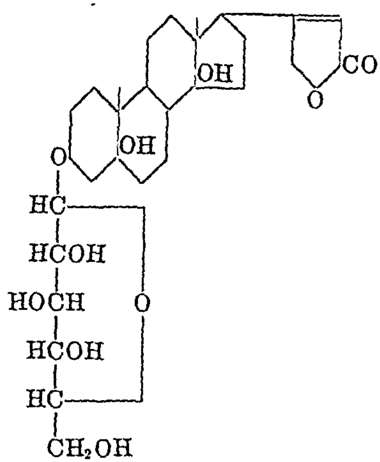
The final products have the following structural formulas:



Digitoxigenin-β-*d*-glucoside



Digoxigenin-β-*d*-glucoside



Periplogenin-β-*d*-glucoside

Biologic assays were carried out with both the tetraacetyl-derivatives and the deacetyl-glucosides. Comparisons were made with the corresponding natural glycosides and their aglycones, namely, digitoxin, digitoxigenin, digoxin, digoxigenin, periplocymarin, and periplogenin. It may be mentioned that periplogenin was studied pharmacologically for the first time in our laboratories.

All the compounds were soluble in dilute ethyl alcohol. A stock solution of 1:1,000 each was prepared with 47.5% ethanol by volume. In etherized cats, the following dilutions were injected intravenously: 1:100,000 for digitoxigenin-, digoxigenin-, and periplogenin-β-*d*-glucosides; 1:25,000 for digitoxigenin- and digoxigenin-β-tetraacetyl-*d*-glucosides; and 1:20,000 for periplogenin. The rate of injection in each case was 1 cc. per minute. In frogs, relatively more concentrated solutions were necessary for injection into the lymph sac, with a minimal amount of alcohol. The following concentrations were employed: 1:500 for periplogenin with 33.25% ethanol; 1:1,000 for digitoxigenin- and digoxigenin-β-tetraacetyl-*d*-glucosides with 47.5% ethanol; 1:2,000 for digitoxin, digoxin, periplogenin-β-*d*-glucoside, and periplocymarin with 31.6%, 24.75%, 23.75%, and 22.17% ethanol, respectively; and 1:4,000 for digitoxigenin- and digoxigenin-β-*d*-glucosides with 25.33% ethanol.

The results in cats, as summarized in table 1, are in accord with those of the strophanthidin series (2). Both digitoxigenin- and digoxigenin-β-*d*-glucosides are more than twice as potent as digitoxin and digoxin, respectively. Periplogenin-β-*d*-glucoside is significantly more active than periplocymarin, although to a lesser degree. Weight for weight, the tetraacetyl-*d*-glucosides of digitoxigenin, digoxigenin, and periplogenin are less potent than their corresponding aglycones, varying approximately from one-third to one-half. Periplocymarin is 4 times as active as periplogenin, further proving the importance of the sugar component of the molecule, as reported previously (5).

TABLE 1
Assay in cats

COMPOUND	SEX	BODY WEIGHT	DOSE TO KILL	MEAN LETHAL DOSE ± STANDARD ERROR
		kgm.	*μgm. per kgm.*	*μgm. per kgm.*
Digitoxin				325.3 ± 11.5*
Digitoxigenin				459.1 ± 36.3†
Digitoxigenin-β-tetraacetyl-*d*-glucoside	F	1.562	1006.4	1184 ±127.1
	M	1.644	1391.7	
	F	1.793	963.7	
	F	1.912	1458.2	
Digitoxigenin-β-*d*-glucoside	F	1.873	123.6	124.7 ± 11.86
	M	1.902	146.2	
	M	1.832	153.4	
	M	2 154	132 3	
	M	1.799	107.3	
	M	2.010	134.8	
	M	1.983	159.4	
	F	2.282	69.2	
Digoxin				235.0 ± 13.96*
Digoxigenin				441.5 ± 41.9†
Digoxigenin-β-tetraacetyl-*d*-glucoside	M	1 894	1129.9	1057 ±146.9
	M	1 737	1038.6	
	F	1.784	733.2	
	M	1.673	1434.5	
Digoxigenin-β-*d*-glucoside	F	2.070	120.3	100.3 ± 5.0
	M	1.759	102 3	
	F	2 456	85.1	
	M	2 432	114.3	
	M	2.681	90.1	
	F	2 630	90.5	
	F	2.335	83.5	
	F	2.312	105.5	
	F	2.292	132.4	
	F	2.193	90.3	
Periplocymarin				153.9 ± 5.5‡
Periplogenin	F	2 186	806 3	719 3 ± 81.3
	F	2.103	646.7	
	F	1 972	682.0	
	F	1 911	981 2	

* Geometrical mean of previously published data (4).
† Geometrical mean of previously published data (5).
‡ This figure is the mean (geometric) of combined data published previously (6, 7, 8).

TABLE 1—*Concluded*

COMPOUND	SEX	BODY WEIGHT	DOSE TO KILL	MEAN LETHAL DOSE ± STANDARD ERROR
		kgm.	*µgm. per kgm.*	*µgm. per kgm.*
Periplogenin—*Concluded*	F	2.001	834.6	
	M	1.980	421.7	
	F	1.830	644.8	
	F	2.022	502.0	
	F	2.277	1297.8	
Periplogenin-β-tetraacetyl-*d*-glucoside	F	2.064	2220.9	2599 ±178
	M	1.902	2268.8	
	F	2.000	2869.1	
	F	2.364	2571.2	
	F	2.233	3186.1	
Periplogenin-β-*d*-glucoside	F	2.095	115.5	118.85 ± 5.79
	M	1.847	132.1	
	M	1.683	127.8	
	M	2.116	95.0	
	F	2.042	120.0	
	F	2.106	126.8	

The results in frogs are shown in table 2. The one-hour method of U.S.P.XI was employed. Since the susceptibility of frogs to digitalis-like drugs varies greatly from lot to lot, far more than that of cats, digitoxin, digoxin, and periplocymarin were simultaneously assayed with the present series of compounds. In other words, comparative tests were made in the same lots of animals and at the same time. Digitoxigenin, digoxigenin, and periplogenin-β-tetraacetyl-*d*-glucoside were the only members that were omitted due to lack of material. Previously published data on the first two substances are listed, however, in table 2 for reference.

In spite of all precautions, the results in frogs are not as uniform as those in cats. Differences in absorption are probably one of the reasons. As in cats, digitoxigenin- and digoxigenin-β-*d*-glucosides are more powerful than digitoxin and digoxin, respectively. Similarly, digitoxigenin-β-tetraacetyl-*d*-glucoside is more active than digitoxigenin, and periplogenin is more active than periplocymarin. It should be pointed out that digitoxigenin-β-tetraacetyl-*d*-glucoside readily crystallized out in the lymph sac, so that the recorded median systolic dose (SD_{50}) very likely underrates its true potency. In contrast with the findings in cats, periplogenin-β-*d*-glucoside proves to be definitely less active than periplocymarin; and digoxigenin, less active than digoxigenin-β-tetraacetyl-*d*-glucoside. The last pair of compounds, as mentioned above, was not studied at the same time. At the present stage of our knowledge, no explanation can be safely advanced to account for the reversal of the order of activity with periplocymarin and periplogenin-β-*d*-glucoside.

TABLE 2
Assay in frogs

COMPOUND	DOSE	NUMBER IN SYSTOLE/NUMBER USED	SD_{50} ± STANDARD ERROR
	µgm. per gm.		*µgm. per gm.*
Digitoxin	5.0	1/5	9.591 ± 0.475
	6.0	3/18	
	7.0	10/25	
	8.0	14/30	
	9.0	16/30	
	10.0	5/5	
	20.0	5/5	
Digitoxigenin			61.93 ± 4.63*
Digitoxigenin-β-tetraacetyl-*d*-glucoside	10.0	0/5	117.5 ±69.4
	20.0	1/5	
	30.0	1/5	
	50.0	1/10	
	100.0	3/5	
Digitoxigenin-β-*d*-glucoside	1.0	0/10	3.934 ± 0.457
	2.0	4/20	
	3.0	6/20	
	4.0	4/10	
	5.0	9/10	
	6.0	3/5	
	9.0	4/5	
Digoxin	2.5	1/5	3.769 ± 0.260
	3.0	3/10	
	4.0	9/20	
	5.0	17/20	
Digoxigenin			33.13 ± 2.52*
Digoxigenin-β-tetraacetyl-*d*-glucoside	8.0	1/10	9.532 ± 0.404
	9.0	4/10	
	10.0	7/10	
Digoxigenin-β-*d*-glucoside	0.7	0/5	2.262 ± 0.256
	0.8	1/5	
	1 0	1/5	
	1.5	1/10	
	2.0	2/10	
	2.5	6/10	
	3.0	12/15	
Periplocymarin	4.0	0/8	5.786 ± 0.416
	6.0	13/20	
	8.0	7/10	

* Median systolic dose of previously published data (5).

TABLE 2—*Concluded*

COMPOUND	DOSE	NUMBER IN SYSTOLE/NUMBER USED	SD_{50} ± STANDARD ERROR
	μgm. per gm.		*μgm. per gm.*
Periplocymarin—*Concluded*	10.0	7/7	
	12.0	7/7	
	14.0	7/7	
Periplogenin	32.0	0/5	85.82 ± 8.06
	60.0	1/5	
	80.0	4/10	
	100.0	8/12	
Periplogenin-β-*d*-glucoside	8.0	0/6	22.07 ± 2.097
	9.0	0/6	
	12.0	1/5	
	15.0	1/7	
	20.0	4/17	
	25.0	5/6	

SUMMARY

1. Six synthetic glycosides, namely, digitoxigenin-β-, digitoxigenin-β-tetraacetyl-, digoxigenin-β-, digoxigenin-β-tetraacetyl-, periplogenin-β-, and periplogenin-β-tetraacetyl-*d*-glucosides, have been studied pharmacologically and compared with their corresponding natural glycosides and aglycones—digitoxin, digitoxigenin, digoxin, digoxigenin, periplocymarin, and periplogenin.

2. In cats, digitoxigenin-, digoxigenin-, and periplogenin-β-*d*-glucosides are more powerful than digitoxin, digoxin, and periplocymarin, respectively. All the tetraacetyl derivatives have a low potency.

3. In frogs, the results are less uniform. While digitoxigenin- and digoxigenin-β-*d*-glucosides are more active than digitoxin and digoxin, respectively, periplogenin-β-*d*-glucoside is weaker than periplocymarin. There is also suggestion that digoxigenin-β-tetraacetyl-*d*-glucoside is more active than digoxigenin.

4. Periplogenin is decidedly less potent than periplocymarin, indicating the favorable influence of the sugar component in the molecule of the glycoside.

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